



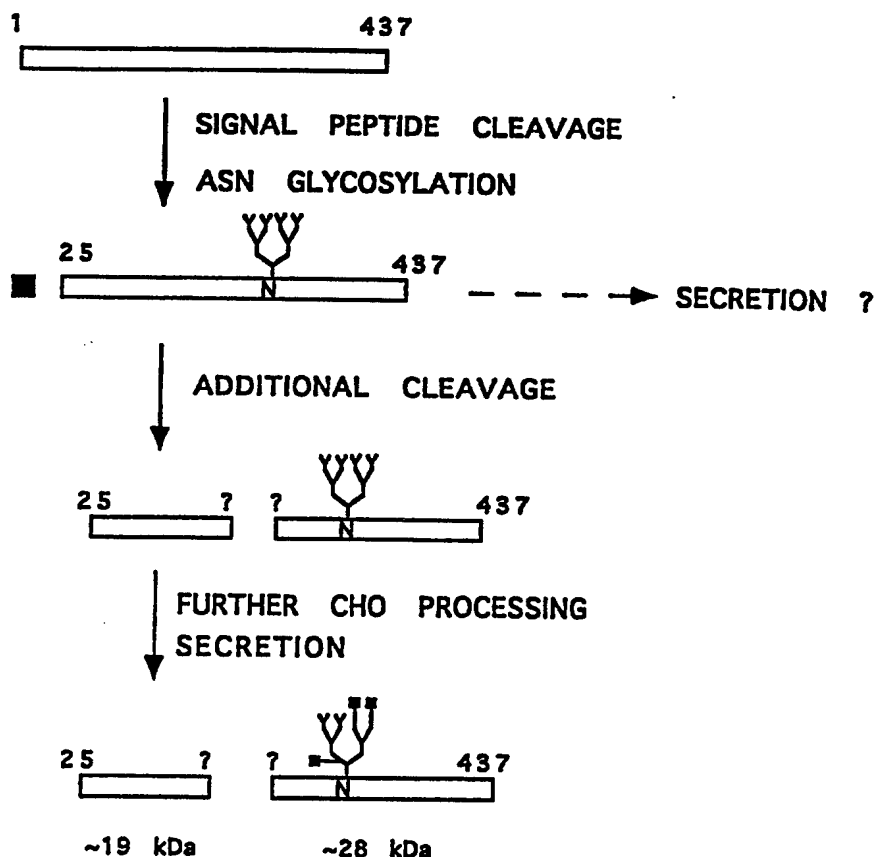
INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : C12N 15/12, C07K 14/46, 14/435, A61K 48/00		A1	(11) International Publication Number: WO 95/18856
			(43) International Publication Date: 13 July 1995 (13.07.95)
(21) International Application Number: PCT/US94/14992		(81) Designated States: AU, CA, JP, KR, NZ, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).	
(22) International Filing Date: 30 December 1994 (30.12.94)		Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>	
(30) Priority Data: 08/176,427 30 December 1993 (30.12.93) US 08/356,060 14 December 1994 (14.12.94) US			
(71) Applicants: PRESIDENT AND FELLOWS OF HARVARD COLLEGE [US/US]; 124 Mt. Auburn Street, Cambridge, MA 02138 (US). IMPERIAL CANCER RESEARCH TECHNOLOGY, LTD. [GB/GB]; Sardinia House, Sardinia Street, London WC2A 3NL (GB).			
(72) Inventors: INGHAM, Philip, W.; 83 Middle Way Road, Summertown, Oxford OX2 7LE (GB). MCMAHON, Andrew, P.; 128 Kendall Road, Lexington, MA 02173 (US). TABIN, Clifford, J.; 102 Hancock Street, Cambridge, MA 02138 (US).			
(74) Agents: VINCENT, Matthew, P. et al.; Lahive & Cockfield, 60 State Street, Boston, MA 02109 (US).			

(54) Title: VERTEBRATE EMBRYONIC PATTERN-INDUCING HEDGEHOG-LIKE PROTEINS

(57) Abstract

The present invention concerns the discovery that proteins encoded by a family of vertebrate genes, termed here *hedgehog*-related genes, comprise morphogenic signals produced by embryonic patterning centers, and are involved in the formation of ordered spatial arrangements of differentiated tissues in vertebrates. The present invention makes available compositions and methods that can be utilized, for example to generate and/or maintain an array of different vertebrate tissue both *in vitro* and *in vivo*.



FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	GB	United Kingdom	MR	Mauritania
AU	Australia	GE	Georgia	MW	Malawi
BB	Barbados	GN	Guinea	NE	Niger
BE	Belgium	GR	Greece	NL	Netherlands
BF	Burkina Faso	HU	Hungary	NO	Norway
BG	Bulgaria	IE	Ireland	NZ	New Zealand
BJ	Benin	IT	Italy	PL	Poland
BR	Brazil	JP	Japan	PT	Portugal
BY	Belarus	KE	Kenya	RO	Romania
CA	Canada	KG	Kyrgyzstan	RU	Russian Federation
CF	Central African Republic	KP	Democratic People's Republic of Korea	SD	Sudan
CG	Congo	KR	Republic of Korea	SE	Sweden
CH	Switzerland	KZ	Kazakhstan	SI	Slovenia
CI	Côte d'Ivoire	LI	Liechtenstein	SK	Slovakia
CM	Cameroon	LK	Sri Lanka	SN	Senegal
CN	China	LU	Luxembourg	TD	Chad
CS	Czechoslovakia	LV	Latvia	TG	Togo
CZ	Czech Republic	MC	Monaco	TJ	Tajikistan
DE	Germany	MD	Republic of Moldova	TT	Trinidad and Tobago
DK	Denmark	MG	Madagascar	UA	Ukraine
ES	Spain	ML	Mali	US	United States of America
FI	Finland	MN	Mongolia	UZ	Uzbekistan
FR	France			VN	Viet Nam
GA	Gabon				

VERTEBRATE EMBRYONIC PATTERN-INDUCING HEDGEHOG-LIKE PROTEINS.

Background of the Invention

5 Pattern formation is the activity by which embryonic cells form ordered spatial
arrangements of differentiated tissues. The physical complexity of higher organisms arises
during embryogenesis through the interplay of cell-intrinsic lineage and cell-extrinsic
signaling. Inductive interactions are essential to embryonic patterning in vertebrate
development from the earliest establishment of the body plan, to the patterning of the organ
10 systems, to the generation of diverse cell types during tissue differentiation (Davidson, E.,
(1990) *Development* 108: 365-389; Gurdon, J. B., (1992) *Cell* 68: 185-199; Jessell, T. M. et
al., (1992) *Cell* 68: 257-270). The effects of developmental cell interactions are varied.
Typically, responding cells are diverted from one route of cell differentiation to another by
inducing cells that differ from both the uninduced and induced states of the responding cells
15 (inductions). Sometimes cells induce their neighbors to differentiate like themselves
(homoiogetic induction); in other cases a cell inhibits its neighbors from differentiating
like itself. Cell interactions in early development may be sequential, such that an initial
induction between two cell types leads to a progressive amplification of diversity. Moreover,
inductive interactions occur not only in embryos, but in adult cells as well, and can act to
20 establish and maintain morphogenetic patterns as well as induce differentiation (J.B. Gurdon
(1992) *Cell* 68:185-199).

The origin of the nervous system in all vertebrates can be traced to the end of
gastrulation. At this time, the ectoderm in the dorsal side of the embryo changes its fate from
epidermal to neural. The newly formed neuroectoderm thickens to form a flattened structure
25 called the neural plate which is characterized, in some vertebrates, by a central groove (neural
groove) and thickened lateral edges (neural folds). At its early stages of differentiation, the
neural plate already exhibits signs of regional differentiation along its anterior posterior (A-P)
and mediolateral axis (M-L). The neural folds eventually fuse at the dorsal midline to form
the neural tube which will differentiate into brain at its anterior end and spinal cord at its
30 posterior end. Closure of the neural tube creates dorsal/ventral differences by virtue of
previous mediolateral differentiation. Thus, at the end of neurulation, the neural tube has a
clear anterior-posterior (A-P), dorsal ventral (D-V) and mediolateral (M-L) polarities (see, for
example, *Principles in Neural Science (3rd)*, eds. Kandel, Schwartz and Jessell, Elsevier
Science Publishing Company: NY, 1991; and *Developmental Biology (3rd)*, ed. S.F. Gilbert,
35 Sinauer Associates: Sunderland MA, 1991). Inductive interactions that define the fate of
cells within the neural tube establish the initial pattern of the embryonic vertebrate nervous
system. In the spinal cord, the identify of cell types is controlled, in part, by signals from two

midline cell groups, the notochord and floor plate, that induce neural plate cells to differentiate into floor plate, motor neurons, and other ventral neuronal types (van Straaten et al. (1988) *Anat. Embryol.* 177:317-324; Placzek et al. (1993) *Development* 117:205-218; Yamada et al. (1991) *Cell* 64:635-647; and Hatta et al. (1991) *Nature* 350:339-341). In addition, signals from the floor plate are responsible for the orientation and direction of commissural neuron outgrowth (Placzek, M. et al., (1990) *Development* 110: 19-30). Besides patterning the neural tube, the notochord and floorplate are also responsible for producing signals which control the patterning of the somites by inhibiting differentiation of dorsal somite derivatives in the ventral regions (Brand-Saberi, B. et al., (1993) *Anat. Embryol.* 188: 239-245; Porquie, O. et al., (1993) *Proc. Natl. Acad. Sci. USA* 90: 5242-5246).

Another important signaling center exists in the posterior mesenchyme of developing limb buds, called the Zone of Polarizing Activity, or "ZPA". When tissue from the posterior region of the limb bud is grafted to the anterior border of a second limb bud, the resultant limb will develop with additional digits in a mirror-image sequence along the anteroposterior axis (Saunders and Gasseling, (1968) *Epithelial-Mesenchymal Interaction*, pp. 78-97). This finding has led to the model that the ZPA is responsible for normal anteroposterior patterning in the limb. The ZPA has been hypothesized to function by releasing a signal, termed a "morphogen", which forms a gradient across the early embryonic bud. According to this model, the fate of cells at different distances from the ZPA is determined by the local concentration of the morphogen, with specific thresholds of the morphogen inducing successive structures (Wolpert, (1969) *Theor. Biol.* 25:1-47). This is supported by the finding that the extent of digit duplication is proportional to the number of implanted ZPA cells (Tickle, (1981) *Nature* 254:199-202).

A candidate for the putative ZPA morphogen was identified by the discovery that a source of retinoic acid can result in the same type of mirror-image digit duplications when placed in the anterior of a limb bud (Tickle et al., (1982) *Nature* 296:564-565; Summerbell, (1983) *J. Embryol* 78:269-289). The response to exogenous retinoic acid is concentration dependent as the morphogen model demands (Tickle et al., (1985) *Dev. Biol.* 109:82-95). Moreover, a differential distribution of retinoic acid exists across the limb bud, with a higher concentration in the ZPA region (Thaller and Eichele, (1987) *Nature* 327:625-628).

Recent evidence, however, has indicated that retinoic acid is unlikely to be the endogenous factor responsible for ZPA activity (reviewed in Brockes, (1991) *Nature* 350:15; Tabin, (1991) *Cell* 66:199-217). It is now believed that rather than directly mimicking an endogenous signal, retinoic acid implants act by inducing an ectopic ZPA. The anterior limb tissue just distal to a retinoic acid implant and directly under the ectoderm has been demonstrated to acquire ZPA activity by serially transplanting that tissue to another limb bud (Summerbell and Harvey, (1983) *Limb Development and Regeneration* pp. 109-118; Wanek

et al., (1991) *Nature* 350:81-83). Conversely, the tissue next to a ZPA graft does not gain ZPA activity (Smith, (1979) *J. Embryol* 52:105-113). Exogenous retinoic acid would thus appear to act upstream of the ZPA in limb patterning.

The immediate downstream targets of ZPA action are not known. However, one important set of genes which are ectopically activated during ZPA-induced pattern duplications are the 5' genes of the Hoxd cluster. These genes are normally expressed in a nested pattern emanating from the posterior margin of the limb bud (Dolle et al., (1989) *Nature* 342:767-772; Izpisua-Belmonte et al., (1991) *Nature* 350:585-589). This nested pattern of Hox gene expression has been directly demonstrated to determine the identity of the structures produced along the anteroposterior axis of the limb (Morgan et al., (1993) *Nature* 358:236-239). As this would predict, ZPA grafts which produce mirror-image duplication of structures at an anatomical level first lead to the ectopic activation of the Hoxd genes in a mirror-image duplication at the molecular level. (Nohno et al., (1991) *Cell* 64:1197-1205; Izpisua-Belmonte et al., (1991) *Nature* 350:585-589). The molecular signals which regulate the expression of these important genes are currently not understood.

Summary of the Invention

The present invention relates to the discovery of a novel family of proteins present in vertebrate organisms, referred to hereinafter as "*hedgehog*" proteins, which proteins have apparent broad involvement in the formation and maintenance of ordered spatial arrangements of differentiated tissues in vertebrates, and can be used to generate and/or maintain an array of different vertebrate tissue both *in vitro* and *in vivo*.

In general, the invention features *hedgehog* polypeptides, preferably substantially pure preparations of one or more of the subject *hedgehog* polypeptides. The invention also provides recombinantly produced *hedgehog* polypeptides. In preferred embodiments the polypeptide has a biological activity including: an ability to modulate proliferation, survival and/or differentiation of mesodermally-derived tissue, such as tissue derived from dorsal mesoderm; the ability to modulate proliferation, survival and/or differentiation of ectodermally-derived tissue, such as tissue derived from the neural tube, neural crest, or head mesenchyme; the ability to modulate proliferation, survival and/or differentiation of endodermally-derived tissue, such as tissue derived from the primitive gut. Moreover, in preferred embodiments, the subject *hedgehog* proteins have the ability to induce expression of secondary signaling molecules, such as members of the Transforming Growth Factor β family, as well as members of the fibroblast growth factor (FGF) family.

4

In a certain embodiments, the polypeptide is identical with or homologous to a *Sonic hedgehog* (*Shh*) polypeptide, such as a mammalian *Shh* represented by SEQ ID Nos: 13 or 11, an avian *Shh* represented by SEQ ID No: 8, or a fish *Shh* represented by SEQ ID No: 12. For instance, the *Shh* polypeptide preferably has an amino acid sequence at least 70% homologous to a polypeptide represented by any of SEQ ID Nos: 8, 11, 12 or 13, though polypeptides with higher sequence homologies of, for example, 80%, 90% or 95% are also contemplated. Exemplary *Shh* proteins are represented by SEQ ID No. 40. The *Shh* polypeptide can comprise a full length protein, such as represented in the sequence listings, or it can comprise a fragment of, for instance, at least 5, 10, 20, 50, 100 or 150 amino acids in length. Preferred *hedgehog* polypeptides include *Shh* sequences corresponding approximately to the natural proteolytic fragments of the *hedgehog* proteins, such as from about Cys-24 through Glu-188, or from about Asn-189 through Ala-475 of the human *Shh* protein, or analogous fragments thereto.

In other embodiments, the polypeptide is identical with or homologous to an *Indian hedgehog* (*Ihh*) polypeptide, such as a human *Ihh* represented by SEQ ID No: 14, or a mouse *Ihh* represented by SEQ ID No: 10. For instance, the *Ihh* polypeptide preferably has an amino acid sequence at least 70% homologous to a polypeptide represented by either of SEQ ID Nos: 10 or 14, though *Ihh* polypeptides with higher sequence homologies of, for example, 80%, 90% or 95% are also contemplated. The polypeptide can comprise the full length protein represented by in part by these sequences, or it can comprise a fragment of, for instance, at least 5, 10, 20, 50, 100 or 150 amino acids in length. Preferred *Ihh* polypeptides comprise an N-terminal fragment including Arg-1 through Glu-94, or a C-terminal fragment including His-95 through Ser-3312 of the human *Ihh* represented by SEQ ID No: 14, or analogous fragments thereto.

In still further embodiments, the polypeptide is identical with or homologous to a *Desert hedgehog* (*Dhh*) polypeptide, such as a mouse *Dhh* represented by SEQ ID No: 9. For instance, the *Dhh* polypeptide preferably has an amino acid sequence at least 70% homologous to a polypeptide represented by SEQ ID No: 9, though *Dhh* polypeptides with higher sequence homologies of, for example, 80%, 90% or 95% are also contemplated. The polypeptide can comprise the full length protein represented by this sequence, or it can comprise a fragment of, for instance, at least 5, 10, 20, 50, 100 or 150 amino acids in length. Preferred *Dhh* polypeptides comprise *Dhh* sequences corresponding to the N-terminal portion of the protein, e.g. Cys-23 through Asp-189 or Asn-190 through Gly-396 of SEQ ID No: 9, or analogous fragments thereto.

Moreover, as described below, the *hedgehog* polypeptide can be either an agonist (e.g. mimics), or alternatively, an antagonist of a biological activity of a naturally occurring form of the protein, e.g., the polypeptide is able to modulate differentiation and/or growth

5

and/or survival of a cell responsive to authentic *hedgehog* proteins. Homologs of the subject *hedgehog* proteins include versions of the protein which are resistant to proteolytic cleavage, as for example, due to mutations which alter potential cleavage sequences or which inactivate an enzymatic activity associated with the protein.

5 The *hedgehog* polypeptides of the present invention can be glycosylated, or conversely, by choice of the expression system or by modification of the protein sequence to preclude glycosylation, reduced carbohydrate analogs can also be provided. Glycosylated forms include derivatization with glycosaminoglycan chains. Likewise, *hedgehog* polypeptides can be generated which lack an endogenous signal sequence (though this is
10 typically cleaved off even if present in the pro-form of the protein).

 The subject proteins can also be provided as chimeric molecules, such as in the form of fusion proteins. For instance, the *hedgehog* protein can be provided as a recombinant fusion protein which includes a second polypeptide portion, e.g., a second polypeptide having an amino acid sequence unrelated to *hedgehog*, e.g. the second polypeptide portion is
15 glutathione-S-transferase, e.g. the second polypeptide portion is an enzymatic activity such as alkaline phosphatase, e.g. the second polypeptide portion is an epitope tag.

 Yet another aspect of the present invention concerns an immunogen comprising a *hedgehog* polypeptide in an immunogenic preparation, the immunogen being capable of eliciting an immune response specific for a *hedgehog* polypeptide; e.g. a humoral response,
20 e.g. an antibody response; e.g. a cellular response. In preferred embodiments, the immunogen comprising an antigenic determinant, e.g. a unique determinant, from a protein represented by one of SEQ ID Nos. 8-14.

 A still further aspect of the present invention features antibodies and antibody preparations specifically reactive with an epitope of the *hedgehog* immunogen.

25 Another aspect of the present invention provides a substantially isolated nucleic acid having a nucleotide sequence which encodes a *hedgehog* polypeptide. In preferred embodiments, the encoded polypeptide specifically agonizes or antagonizes inductive events mediated by wild-type *hedgehog* proteins. The coding sequence of the nucleic acid can comprise a sequence which is identical to a coding sequence represented in one of SEQ ID
30 Nos: 1-7, or it can merely be homologous to one or more of those sequences. For instance, the *hedgehog* encoding sequence preferably has a sequence at least 70% homologous to a nucleotide sequence in one or more of SEQ ID Nos: 1-7, though higher sequence homologies of, for example, 80%, 90% or 95% are also contemplated. The polypeptide encoded by the nucleic acid can comprise an amino acid sequence represented in one of SEQ ID Nos: 8-14
35 such as one of those full length proteins, or it can comprise a fragment of that nucleic acid, which fragment may, for instance, encode a fragment which is, for example, at least 5, 10, 20,

50 or 100 amino acids in length. The polypeptide encoded by the nucleic acid can be either an agonist (e.g. mimics), or alternatively, an antagonist of a biological activity of a naturally occurring form of a *hedgehog* protein.

Furthermore, in certain preferred embodiments, the subject *hedgehog* nucleic acid will include a transcriptional regulatory sequence, e.g. at least one of a transcriptional promoter or transcriptional enhancer sequence, which regulatory sequence is operably linked to the *hedgehog* gene sequence. Such regulatory sequences can be used in to render the *hedgehog* gene sequence suitable for use as an expression vector.

In yet a further preferred embodiment, the nucleic acid hybridizes under stringent conditions to a nucleic acid probe corresponding to at least 12 consecutive nucleotides of one or more of SEQ ID Nos: 1-7; though preferably corresponding to at least 20 consecutive nucleotides; and more preferably corresponding to at least 40, 50 or 75 consecutive nucleotides of one or more of SEQ ID Nos: 1-7.

The invention also features transgenic non-human animals, e.g. mice, rats, rabbits, chickens, frogs or pigs, having a transgene, e.g., animals which include (and preferably express) a heterologous form of a *hedgehog* gene described herein, or which misexpress an endogenous *hedgehog* gene, e.g., an animal in which expression of one or more of the subject *hedgehog* proteins is disrupted. Such a transgenic animal can serve as an animal model for studying cellular and tissue disorders comprising mutated or mis-expressed *hedgehog* alleles or for use in drug screening.

The invention also provides a probe/primer comprising a substantially purified oligonucleotide, wherein the oligonucleotide comprises a region of nucleotide sequence which hybridizes under stringent conditions to at least 10 consecutive nucleotides of sense or antisense sequence of SEQ ID No: 1, or naturally occurring mutants thereof. Nucleic acid probes which are specific for each of the classes of vertebrate *hedgehog* proteins are contemplated by the present invention, e.g. probes which can discern between nucleic acid encoding an *Shh* versus an *Ihh* versus a *Dhh* versus an *Mhh*. In preferred embodiments, the probe/primer further includes a label group attached thereto and able to be detected. The label group can be selected, e.g., from a group consisting of radioisotopes, fluorescent compounds, enzymes, and enzyme co-factors. Probes of the invention can be used as a part of a diagnostic test kit for identifying dysfunctions associated with mis-expression of a *hedgehog* protein, such as for detecting in a sample of cells isolated from a patient, a level of a nucleic acid encoding a subject *hedgehog* protein; e.g. measuring a *hedgehog* mRNA level in a cell, or determining whether a genomic *hedgehog* gene has been mutated or deleted. Preferably, the oligonucleotide is at least 10 nucleotides in length, though primers of 20, 30, 50, 100, or 150 nucleotides in length are also contemplated.

In yet another aspect, the invention provides an assay for screening test compounds for inhibitors, or alternatively, potentiators, of an interaction between a *hedgehog* protein and a *hedgehog* receptor. An exemplary method includes the steps of (i) combining a *hedgehog* receptor, either soluble or membrane bound (including whole cells), a *hedgehog* polypeptide, and a test compound, e.g., under conditions wherein, but for the test compound, the *hedgehog* protein and the *hedgehog* receptor are able to interact; and (ii) detecting the formation of a complex which includes the *hedgehog* protein and the receptor either by directly quantitating the complex or by measuring inductive effects of the *hedgehog* protein. A statistically significant change, such as a decrease, in the formation of the complex in the presence of a test compound (relative to what is seen in the absence of the test compound) is indicative of a modulation, e.g., inhibition, of the interaction between the *hedgehog* protein and the receptor.

Another aspect of the present invention relates to a method of inducing and/or maintaining a differentiated state, causing proliferation, and/or enhancing survival of a cell (from a vertebrate organism) responsive to a *hedgehog* protein, by contacting the cells with a *hedgehog* agonist. For example, the present method is applicable to cell culture technique, such as in the culturing of neuronal and other cells whose survival or differentiative state is dependent on *hedgehog* function. Moreover, *hedgehog* agonists and antagonists can be used for therapeutic intervention, such as to enhance survival and maintenance of neurons and other neural cells in both the central nervous system and the peripheral nervous system, as well as to influence other vertebrate organogenic pathways, such as other ectodermal patterning, as well as certain mesodermal and endodermal differentiation processes. In addition to the vertebrate *hedgehog*-like proteins, the present invention further contemplates the use of *Drosophila* Hedgehog (*Dros*-HH) to induce cells and tissue of vertebrate organisms in similar fashion to the subject *hedgehog* proteins.

Another aspect of the present invention provides a method of determining if a subject, e.g. a human patient, is at risk for a disorder characterized by unwanted cell proliferation or aberrant control of differentiation. The method includes detecting, in a tissue of the subject, the presence or absence of a genetic lesion characterized by at least one of (i) a mutation of a gene encoding a *hedgehog* protein, e.g. represented in SEQ ID No: 2, or a homolog thereof; or (ii) the mis-expression of a *hedgehog* gene. In preferred embodiments, detecting the genetic lesion includes ascertaining the existence of at least one of: a deletion of one or more nucleotides from a *hedgehog* gene; an addition of one or more nucleotides to the gene, a substitution of one or more nucleotides of the gene, a gross chromosomal rearrangement of the gene; an alteration in the level of a messenger RNA transcript of the gene; the presence of a non-wild type splicing pattern of a messenger RNA transcript of the gene; or a non-wild type level of the protein.

For example, detecting the genetic lesion can include (i) providing a probe/primer including an oligonucleotide containing a region of nucleotide sequence which hybridizes to a sense or antisense sequence of a *hedgehog* gene, e.g. a nucleic acid represented in one of SEQ ID Nos: 1-7, or naturally occurring mutants thereof, or 5' or 3' flanking sequences naturally associated with the *hedgehog* gene; (ii) exposing the probe/primer to nucleic acid of the tissue; and (iii) detecting, by hybridization of the probe/primer to the nucleic acid, the presence or absence of the genetic lesion; e.g. wherein detecting the lesion comprises utilizing the probe/primer to determine the nucleotide sequence of the *hedgehog* gene and, optionally, of the flanking nucleic acid sequences. For instance, the probe/primer can be employed in a polymerase chain reaction (PCR) or in a ligation chain reaction (LCR). In alternate embodiments, the level of a *hedgehog* protein is detected in an immunoassay using an antibody which is specifically immunoreactive with the *hedgehog* protein.

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of cell biology, cell culture, molecular biology, transgenic biology, microbiology, recombinant DNA, and immunology, which are within the skill of the art. Such techniques are explained fully in the literature. See, for example, *Molecular Cloning A Laboratory Manual*, 2nd Ed., ed. by Sambrook, Fritsch and Maniatis (Cold Spring Harbor Laboratory Press: 1989); *DNA Cloning*, Volumes I and II (D. N. Glover ed., 1985); *Oligonucleotide Synthesis* (M. J. Gait ed., 1984); Mullis et al. U.S. Patent No: 4,683,195; *Nucleic Acid Hybridization* (B. D. Hames & S. J. Higgins eds. 1984); *Transcription And Translation* (B. D. Hames & S. J. Higgins eds. 1984); *Culture Of Animal Cells* (R. I. Freshney, Alan R. Liss, Inc., 1987); *Immobilized Cells And Enzymes* (IRL Press, 1986); B. Perbal, *A Practical Guide To Molecular Cloning* (1984); the treatise, *Methods In Enzymology* (Academic Press, Inc., N.Y.); *Gene Transfer Vectors For Mammalian Cells* (J. H. Miller and M. P. Calos eds., 1987, Cold Spring Harbor Laboratory); *Methods In Enzymology*, Vols. 154 and 155 (Wu et al. eds.), *Immunochemical Methods In Cell And Molecular Biology* (Mayer and Walker, eds., Academic Press, London, 1987); *Handbook Of Experimental Immunology*, Volumes I-IV (D. M. Weir and C. C. Blackwell, eds., 1986); *Manipulating the Mouse Embryo*, (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1986).

Other features and advantages of the invention will be apparent from the following detailed description, and from the claims.

Brief Description of the Drawings

Figures 1 represents the amino acid sequences of two chick *hh* clones, chicken *hedgehog*-A (pCHA; SEQ ID No:35) and chicken *hedgehog*-B (pCHB; SEQ ID No:36). These clones were obtained using degenerate primers corresponding to the underlined amino

acid residues of the *Drosophila* sequence (corresponding to residues 161-232 of SEQ ID No:34) also shown in Figure 1, followed by nested PCR using chicken genomic DNA.

Figure 2 is an alignment comparing the amino acid sequences of chick *Shh* (SEQ ID No:8) with its *Drosophila* homolog (SEQ ID No:34). *Shh* residues 1-26 correspond to the proposed signal peptide. Identical residues are enclosed by boxes and gaps in order to highlight similarity. The nucleotide sequence of *Shh* has been submitted to Genbank.

Figure 3 is a hydropathy plot for the predicted chick *Shh* protein, generated by the methods of Kyte and Doolittle (1982). The values of hydrophobicity are plotted against the amino acid positions. Negative values predict a hydrophobic domain of the protein.

Figure 4 is an alignment comparing the amino acid sequences of various *hh* proteins. The white region on the amino terminus of chicken *Shh* corresponds to the putative signal peptide. The black box refers to a highly conserved region from aa residues 26-207 of SEQ ID No:8). The arrows point to exon boundaries in the *Drosophila* gene (Lee et al. (1992) *Cell* 71: 33-50). In each case, the proteins are compared to chicken *Shh* (SEQ ID No:8) and the percent amino acid identity is indicated in each region's box.

Figure 5A is a "pileup" alignment of predicted amino acid sequences which compares *Drosophila hh* (D-*hh*; SEQ ID No:34), mouse *hh* (M-*Dhh*; SEQ ID No:9; M-*Ihh*; SEQ ID No:10; M-*Shh*; SEQ ID No:11), chicken *hh* (C-*Shh*; SEQ ID No:8), and zebrafish *hh* (Z-*Shh*; SEQ ID No:12). The predicted hydrophobic transmembrane/signal sequences are indicated in italics and the predicted signal sequence processing site is arrowed. The positions of introns interrupting the *Drosophila hh* and M-*Dhh* open reading frames are indicated by arrowheads. All amino acids shared among the six predicted *hh* proteins are indicated in bold. Figure 5B is a sequence alignment of the N-terminal portion of vertebrate *hedgehog* proteins, and the predicted degenerate sequence "CON" (SEQ ID No: 41).

Figure 6 is an inter- and cross-species comparison of amino acid identities among the predicted processed *hh* proteins shown in Figure 5A. All values are percentages. Figures in parentheses represent similarities allowing for conservative amino acid substitutions.

Figure 7 is a representation of the DNA constructs used in transgenic studies to study ectopic expression of chick *Shh* in mouse embryos. Constructs were generated for ectopic expression of cDNA clones in the *Wnt-1* expression domain and tested in transgenic mice embryos using a lac-Z reporter (pWEXP-*lacZ* (used as a control)) and a chick *Shh* reporter (pWEXP-CShh). The pWEXP-CShh construct contained two tandem head to tail copies of a chick *Shh* cDNA. The results of WEXP2-CShh transgenic studies are shown in Table 1.

Figure 8 is a model for anterioposterior limb patterning and the Zone of Polarizing Activity (ZPA), based on Saunders and Gasseling (1968). The left portion of the diagram

10

schematizes a stage 20 limb bud. The somites are illustrated as blocks along the left margin of the limb bud; right portion of the same panel illustrates the mature wing. The hatched region on the posterior limb is the ZPA. Normally, the developed wing contains three digits II, III, and IV. The figure further shows the result of transplanting a ZPA from one limb bud to the anterior margin of another. The mature limb now contains six digits IV, III, II, II, III, and IV in a mirror-image duplication of the normal pattern. The large arrows in both panels represent the signal produced by the ZPA which acts to specify digit identity.

Figures 9A and 9B illustrate the comparison of zebrafish *Shh* (Z-*Shh*) and *Drosophila hh* (hh) amino acid sequences. Figure 9A is an alignment of zebrafish *Shh* and *Drosophila hh* amino acid sequences. Identical amino acids are linked by vertical bars. Dots indicate gaps introduced for optimal alignment. Putative transmembrane/signal peptide sequences are underlined (Kyte and Doolittle (1982) *J Mol Biol* 157:133-148). The position of exon boundaries in the *Drosophila* gene are indicated by arrowheads. The region of highest similarity between Z-*Shh* and *hh* overlaps exon 2. Figure 9B is a schematic comparison of Z-*Shh* and *Drosophila hh*. Black boxes indicate the position of the putative transmembrane/signal peptide sequences, relative to the amino-terminus. Sequence homologies were scored by taking into account the alignment of chemically similar amino acids and percentage of homology in the boxed regions is indicated.

Figure 10 is an alignment of partial predicted amino acid sequences from three different zebrafish *hh* homologs. One of these sequences corresponds to *Shh*, while the other two define additional *hh* homologs in zebrafish, named *hh(a)* and *hh(b)*. Amino acid identities among the three partial homologs are indicated by vertical bars.

Figure 11 is a schematic representations of chick and mouse *Shh* proteins. The putative signal peptides and Asn-linked glycosylation sites are shown. The numbers refer to amino acid positions.

Figure 12 is a schematic representation of myc-tagged *Shh* constructs. The positions of the c-myc epitope tags are shown, as is the predicted position of the proteolytic cleavage site. The shaded area following the signal peptide of the carboxy terminal tagged construct represents the region included in the Glutathione-S-transferase fusion protein used to generate antisera in rabbits.

Figure 13 is a schematic diagram of *Shh* processing. Illustrated are cleavage of the signal peptide (black box), glycosylation at the predicted Asn residue (N), and the secondary proteolytic cleavage. The question marks indicate that the precise site of proteolytic cleavage has not been determined. The different symbols representing the carbohydrate moiety indicated maturation of this structure in the Golgi apparatus. The dashed arrow leading from

11

the signal peptide cleaved protein indicates that secretion of this species may be an artifact of the incomplete proteolytic processing of *Shh* seen in *Xenopus* oocytes and cos cells.

Figure 14 is a schematic diagram of a model for the coordinated growth and patterning of the limb. *Sonic* is proposed to signal directly to the mesoderm to induce expression of the *Hoxd* and *Bmp-2* genes. The induction of these mesodermal genes requires competence signals from the overlying AER. One such signal is apparently *Fgf-4*. Expression of *Fgf-4* in the AER can be induced by *Sonic* providing an indirect signaling pathway from *Sonic* to the mesoderm. FGFs also maintain expression of *Sonic* in the ZPA, thereby completing a positive feedback loop which controls the relative positions of the signaling centers. While *Fgf-4* provides competence signals to the mesoderm, it also promotes mesodermal proliferation. Thus patterning of the mesoderm is dependent on the same signals which promote its proliferation. This mechanism inextricably integrates limb patterning with outgrowth.

Figure 15 is a schematic diagram of patterning of the *Drosophila* and vertebrate gut. Regulatory interactions responsible for patterning of *Drosophila* midgut (A) are compared to a model for patterning of the vertebrate hindgut (B) based on expression data. Morphologic regional distinctions are indicated to the left (A and B), genes expressed in the visceral mesoderm are in the center panel, those in the gut luminal endoderm are on the right. *HOM/Hox* gene expression domains are boxed. Regionally expressing secreted gene products are indicated by lines. Arrows indicate activating interactions, barred lines, inhibiting interactions. Regulatory interactions in *Drosophila* gut (A) have been established by genetic studies except for the relationship between *dpp* and *hedgehog*, which is hypothesized based on their interactions in the *Drosophila* imaginal discs, *hedgehog* appears to be a signal from the endoderm to the mesoderm, and that *dpp* is expressed in the mesoderm.

Figure 16 is a schematic diagram of chromosomal locations of *Ihh*, *Shh* and *Dhh* in the mouse genome. The loci were mapped by interspecific backcross analysis. The segregation patterns of the loci and flanking genes in backcross animals that were typed for all loci are shown above the chromosome maps. For individual pairs of loci more animals were typed. Each column represents the chromosome identified in the backcross progeny that was inherited from the (C57BL/6J x *M. spretus*) F1 parent. The shaded boxes represent the presence of a C57BL/6J allele and white boxes represent the presence of a *M. spretus* allele. The number of the offsprings inheriting each type of chromosome is listed at the bottom of each column. Partial chromosome linkage maps showing location of *Ihh*, *Shh* and *Dhh* in relation too linked genes is shown. The number of recombinant N₂ animals is

presented over total number of N_2 animals typed to the left of the chromosome maps between each pair of loci. The recombinant frequencies, expressed as genetic distance in centimorgans (\pm one standard error) are also shown. When no recombination between loci was detected, the upper 95% confidence limit of the recombination distance is indicated in parentheses. Gene order was determined by minimizing the number of recombinant events required to explain the allele distribution patterns. The position of loci in human chromosomes can be obtained from GDB (Genome Data Base), a computerized database of human linkage information maintained by the William H. Welch Medical Library of the John Hopkins University (Baltimore, MD).

Detailed Description of the Invention

Embryonic inductive signals are key regulatory proteins that function in vertebrate pattern formation, and are present in important signaling centers known to operate embryonically to define the organization of the vertebrate embryo. For example, these signaling structures include the notochord, a transient structure which initiates the formation of the nervous system and helps to define the different types of neurons within it. The notochord also regulates mesodermal patterning along the body axis. Another distinct group of cells having apparent signaling activity is the floorplate of the neural tube (the precursor of the spinal cord and brain) which also signals the differentiation of different nerve cell types. It is also generally believed that the region of mesoderm at the bottom of the buds which form the limbs (called the Zone of Polarizing Activity or ZPA) operates as a signaling center by secreting a morphogen which ultimately produces the correct patterning of the developing limbs.

The present invention concerns the discovery that proteins encoded by a family of vertebrate genes, termed here *hedgehog*-related genes, comprise the signals produced by these embryonic patterning centers. As described herein, each of the disclosed vertebrate *hedgehog* (*hh*) homologs exhibits spatially and temporally restricted expression domains indicative of important roles in embryonic patterning. For instance, the results provided below indicate that vertebrate *hh* genes are expressed in the posterior limb bud, Hensen's node, the early notochord, the floor plate of the neural tube, the fore- and hindgut and their derivatives. These are all important signaling centers known to be required for proper patterning of surrounding embryonic tissues.

The *Hedgehog* family of vertebrate inter-cellular signaling molecules provided by the present invention consists of at least four members. Three of these members, herein referred to as Desert *hedgehog* (*Dhh*), Sonic *hedgehog* (*Shh*) and Indian *hedgehog* (*Ihh*), exist in all vertebrates, including fish, birds, and mammals. A fourth member, herein referred to as

13

Moonrat *hedgehog* (*Mhh*), appears specific to fish. According to the appended sequence listing, (see also Table 1) a chicken *Shh* polypeptide is encoded by SEQ ID No:1; a mouse *Dhh* polypeptide is encoded by SEQ ID No:2; a mouse *Ihh* polypeptide is encoded by SEQ ID No:3; a mouse *Shh* polypeptide is encoded by SEQ ID No:4 a zebrafish *Shh* polypeptide is encoded by SEQ ID No:5; a human *Shh* polypeptide is encoded by SEQ ID No:6; and a human *Ihh* polypeptide is encoded by SEQ ID No:7.

Table 1
Guide to vertebrate *hedgehog* sequences

	Nucleotide	Amino Acid
Chicken <i>Shh</i>	SEQ ID No. 1	SEQ ID No. 8
Mouse <i>Dhh</i>	SEQ ID No. 2	SEQ ID No. 9
Mouse <i>Ihh</i>	SEQ ID No. 3	SEQ ID No. 10
Mouse <i>Shh</i>	SEQ ID No. 4	SEQ ID No. 11
Zebrafish <i>Shh</i>	SEQ ID No. 5	SEQ ID No. 12
Human <i>Shh</i>	SEQ ID No. 6	SEQ ID No. 13
Human <i>Ihh</i>	SEQ ID No. 7	SEQ ID No. 14

Certain of the vertebrate *Hedgehog* proteins (*hh*) of the present invention are defined by SEQ ID Nos:8-14 and can be cloned from vertebrate organisms including fish, avian and mammalian sources. These proteins are distinct from the *Drosophila* protein referred to in the literature as a hedgehog protein which, for clarity, will be referred to hereinafter as "Dros-HH". In addition to the sequence variation between the various *hh* homologs, the vertebrate *hedgehog* proteins are apparently present naturally in a number of different forms, including a pro-form, a full-length mature form, and several processed fragments thereof. The pro-form includes an N-terminal signal peptide for directed secretion of the extracellular domain, while the full-length mature form lacks this signal sequence. Further processing of the mature form apparently occurs in some instances to yield biologically active fragments of the protein. For instance, *sonic hedgehog* undergoes additional proteolytic processing to yield two peptides of approximately 19 kDa and 27 kDa, both of which are secreted. In addition to proteolytic fragmentation, the vertebrate *hedgehog* proteins can also be modified post-translationally, such as by glycosylation, though bacterially produced (e.g. unglycosylated) forms of the proteins apparently still maintain at least some of the activity of the native protein.

As described in the following examples, the cDNA clones provided by the present invention were first obtained by screening a mouse genomic library with a partial *Drosophila hh* cDNA clone (.7kb). Positive plaques were identified and one mouse clone was selected. This clone was then used as a probe to obtain a genomic clone containing the full coding sequence of the Mouse *Dhh* gene. As described in the attached Examples, Northern blots and

in situ hybridization demonstrated that Mouse *Dhh* is expressed in the testes, and potentially the ovaries, and is also associated with sensory neurons of the head and trunk. *Dhh* is clearly a secreted factor expressed by Sertoli cells in the male testes, which is required for maintenance of the male germ line as probably a mitotic and survival factor. *Dhh* mutants are male sterile. Furthermore, *Dhh* is expressed as one of the first signs of differentiation of the gonad, thus *Dhh* may be a target of the sex determining gene, *Sry*. Interestingly, no expression was detected on the nerve cell bodies themselves (only the axons), indicating that *Dhh* is likely produced by the Schwann cells.

In order to obtain cDNA clones encoding chicken *hh* genes, degenerate oligonucleotides were designed corresponding to the amino and carboxy ends of *Drosophila hh* exon 2. As described in the Examples below, these oligonucleotides were used to isolate PCR fragments from chicken genomic DNA. These fragments were then cloned and sequenced. Ten clones yielded two different *hh* homologs, chicken *Dhh* and chicken *Shh*. The chicken *Shh* clone was then used to screen a stage 21/22 limb bud cDNA library which yielded a full length *Shh* clone.

In order to identify other vertebrate *hedgehog* homologs, the chicken clones (*Dhh* and *Shh*) were used to probe a genomic southern blot containing chicken DNA. As described below, genomic DNA was cut with various enzymes which do not cleave within the probe sequences. The DNA was run on a gel and transferred to a nylon filter. Probes were derived by ligating each 220 bp clone into a concatomer and then labeling with a random primer kit. The blots were hybridized and washed at low stringency. In each case, three hybridizing bands were observed following autoradiography, one of which was significantly more intense (a different band with each probe), indicating that there are at least three vertebrate *hh* genes. Additional cDNA and genomic screens carried out have yielded clones of three *hh* homologs from chickens and mice (*Shh*, *Dhh* and *Ihh*), and four *hh* homologs from zebrafish (*Shh*, *Dhh*, *Ihh* and *Mhh*). Weaker hybridization signals suggested that the gene family may be even larger. Moreover, a number of weakly hybridizing genomic clones have been isolated. Subsequently, the same probes derived from chicken *hedgehog* homologs have been utilized to screen a human genomic library. PCR fragments derived from the human genomic library were then sequenced, and PCR probes derived from the human sequences were used to screen human fetal cDNA libraries. Full-length cDNA encoding human sonic *hedgehog* protein (*Shh*) and partial cDNA encoding human Indian *hedgehog* protein (*Ihh*) were isolated from the fetal library, and represent a source of recombinant human *hedgehog* proteins.

To order to determine the expression patterns of the various vertebrate *hh* homologs, *in situ* hybridizations were performed in developing embryos of chicken, mice and fish. As described in the Examples below, the resulting expression patterns of each *hh* homolog were similar across each species and revealed that *hh* genes are expressed in a number of important

embryonic signaling centers. For example, *Shh* is expressed in Hensen's node, the notochord, the ventral floorplate of the developing neural tube, and the ZPA at the base of the limb buds. *Shh* is also expressed in differentiated motor neurons in the embryonic mouse (at 11.5 days post fertilization), therefore, *Shh* may play a role in later stages of motor neuron development, perhaps in proliferation, but more likely in survival of this cell population. *Ihh* is expressed in the embryonic yolk sac and hindgut, and appear also to be involved in chondrogenesis; *Dhh* is expressed in the testes; and *Mhh* (only in zebrafish) is expressed in the notochord and in certain cranial nerves.

Furthermore, experimental evidence indicates that certain *hedgehog* proteins initiate expression of secondary signaling molecules, including *Bmp-2* (a TGF- β relative) in the mesoderm and *Fgf-4* in the ectoderm. The mesoderm requires ectodermally-derived competence factor(s), which include *Fgf-4*, to activate target gene expression in response to *hedgehog* signaling. The expression of, for example, Sonic and *Fgf-4* is coordinately regulated by a positive feedback loop operating between the posterior mesoderm and the overlying AER, which is the ridge of pseudostratified epithelium extending antero-posteriorly along the distal margin of the bud. These data provide a basis for understanding the integration of growth and patterning in the developing limb which can have important implications in the treatment of bone disorders described in greater detail herein.

To determine the role *hedgehog* proteins plays in inductive interactions between the endoderm and mesoderm, which are critical to gut morphogenesis, in situ hybridizations and recombinant retroviral injections were performed in developing chick embryos. The ventral mesoderm is induced to undergo gut-specific differentiation by the adjacent endoderm. As described in Examples below, at the earliest stages of chick gut formation *Shh* is expressed by the endoderm, and *BMP-4* (a TGF- β relative) is expressed in the adjacent visceral mesoderm. Ectopic expression of *Sonic* is sufficient to induce expression of *BMP-4* in visceral mesoderm, suggesting that *Sonic* serves as an inductive signal from the endoderm to the mesoderm. Subsequent organ-specific endodermal differentiation depends on regional inductive signal from the visceral mesoderm. Hox genes are expressed in the undifferentiated chick hind gut mesoderm with boundaries corresponding to morphologic borders, suggesting a role in regulating gut morphogenesis.

Accordingly, certain aspects of the present invention relate to nucleic acids encoding vertebrate *hedgehog* proteins, the *hedgehog* proteins themselves, antibodies immunoreactive with *hh* proteins, and preparations of such compositions. Moreover, the present invention provides diagnostic and therapeutic assays and reagents for detecting and treating disorders involving, for example, aberrant expression of vertebrate *hedgehog* homologs. In addition, drug discovery assays are provided for identifying agents which can modulate the binding of vertebrate *hedgehog* homologues to *hedgehog*-binding moieties (such as *hedgehog* receptors,

ligands, or other extracellular matrix components). Such agents can be useful therapeutically to alter the growth and/or differentiation of a cell. Other aspects of the invention are described below or will be apparent to those skilled in the art in light of the present disclosure.

5 For convenience, certain terms employed in the specification, examples, and appended claims are collected here.

As used herein, the term "nucleic acid" refers to polynucleotides such as deoxyribonucleic acid (DNA), and, where appropriate, ribonucleic acid (RNA). The term should also be understood to include, as equivalents, analogs of either RNA or DNA made
10 from nucleotide analogs, and, as applicable to the embodiment being described, single (sense or antisense) and double-stranded polynucleotides.

As used herein, the term "gene" or "recombinant gene" refers to a nucleic acid comprising an open reading frame encoding one of the vertebrate *hh* polypeptides of the present invention, including both exon and (optionally) intron sequences. A "recombinant
15 gene" refers to nucleic acid encoding a vertebrate *hh* polypeptide and comprising vertebrate *hh*-encoding exon sequences, though it may optionally include intron sequences which are either derived from a chromosomal vertebrate *hh* gene or from an unrelated chromosomal gene. Exemplary recombinant genes encoding the subject vertebrate *hh* polypeptide are represented by SEQ ID No:1, SEQ ID No:2, SEQ ID No:3, SEQ ID No:4, SEQ ID No:5,
20 SEQ ID No:6 or SEQ ID No:7. The term "intron" refers to a DNA sequence present in a given vertebrate *hh* gene which is not translated into protein and is generally found between exons.

As used herein, the term "transfection" means the introduction of a nucleic acid, e.g., an expression vector, into a recipient cell by nucleic acid-mediated gene transfer.
25 "Transformation", as used herein, refers to a process in which a cell's genotype is changed as a result of the cellular uptake of exogenous DNA or RNA, and, for example, the transformed cell expresses a recombinant form of a vertebrate *hh* polypeptide or, where anti-sense expression occurs from the transferred gene, the expression of a naturally-occurring form of the vertebrate *hh* protein is disrupted.

30 As used herein, the term "vector" refers to a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked. One type of preferred vector is an episome, i.e., a nucleic acid capable of extra-chromosomal replication. Preferred vectors are those capable of autonomous replication and/or expression of nucleic acids to which they are linked. Vectors capable of directing the expression of genes to which they are operatively
35 linked are referred to herein as "expression vectors". In general, expression vectors of utility in recombinant DNA techniques are often in the form of "plasmids" which refer generally to

circular double stranded DNA loops which, in their vector form are not bound to the chromosome. In the present specification, "plasmid" and "vector" are used interchangeably as the plasmid is the most commonly used form of vector. However, the invention is intended to include such other forms of expression vectors which serve equivalent functions and which become known in the art subsequently hereto.

"Transcriptional regulatory sequence" is a generic term used throughout the specification to refer to DNA sequences, such as initiation signals, enhancers, and promoters, which induce or control transcription of protein coding sequences with which they are operably linked. In preferred embodiments, transcription of one of the recombinant vertebrate *hedgehog* genes is under the control of a promoter sequence (or other transcriptional regulatory sequence) which controls the expression of the recombinant gene in a cell-type in which expression is intended. It will also be understood that the recombinant gene can be under the control of transcriptional regulatory sequences which are the same or which are different from those sequences which control transcription of the naturally-occurring forms of *hedgehog* proteins.

As used herein, the term "tissue-specific promoter" means a DNA sequence that serves as a promoter, i.e., regulates expression of a selected DNA sequence operably linked to the promoter, and which effects expression of the selected DNA sequence in specific cells of a tissue, such as cells of neural origin, e.g. neuronal cells. The term also covers so-called "leaky" promoters, which regulate expression of a selected DNA primarily in one tissue, but cause expression in other tissues as well.

As used herein, a "transgenic animal" is any animal, preferably a non-human mammal, bird or an amphibian, in which one or more of the cells of the animal contain heterologous nucleic acid introduced by way of human intervention, such as by transgenic techniques well known in the art. The nucleic acid is introduced into the cell, directly or indirectly by introduction into a precursor of the cell, by way of deliberate genetic manipulation, such as by microinjection or by infection with a recombinant virus. The term genetic manipulation does not include classical cross-breeding, or *in vitro* fertilization, but rather is directed to the introduction of a recombinant DNA molecule. This molecule may be integrated within a chromosome, or it may be extrachromosomally replicating DNA. In the typical transgenic animals described herein, the transgene causes cells to express a recombinant form of one of the vertebrate *hh* proteins, e.g. either agonistic or antagonistic forms. However, transgenic animals in which the recombinant vertebrate *hh* gene is silent are also contemplated, as for example, the FLP or CRE recombinase dependent constructs described below. The "non-human animals" of the invention include vertebrates such as rodents, non-human primates, sheep, dog, cow, chickens, amphibians, reptiles, etc. Preferred non-human animals are selected from the rodent family including rat and mouse, most

preferably mouse, though transgenic amphibians, such as members of the *Xenopus* genus, and transgenic chickens can also provide important tools for understanding and identifying agents which can affect, for example, embryogenesis and tissue formation. The term "chimeric animal" is used herein to refer to animals in which the recombinant gene is found, or in which
5 the recombinant is expressed in some but not all cells of the animal. The term "tissue-specific chimeric animal" indicates that one of the recombinant vertebrate *hh* genes is present and/or expressed in some tissues but not others.

As used herein, the term "transgene" means a nucleic acid sequence (encoding, e.g., one of the vertebrate *hh* polypeptides), which is partly or entirely heterologous, i.e., foreign,
10 to the transgenic animal or cell into which it is introduced, or, is homologous to an endogenous gene of the transgenic animal or cell into which it is introduced, but which is designed to be inserted, or is inserted, into the animal's genome in such a way as to alter the genome of the cell into which it is inserted (e.g., it is inserted at a location which differs from that of the natural gene or its insertion results in a knockout). A transgene can include one or
15 more transcriptional regulatory sequences and any other nucleic acid, such as introns, that may be necessary for optimal expression of a selected nucleic acid.

As is well known, genes for a particular polypeptide may exist in single or multiple copies within the genome of an individual. Such duplicate genes may be identical or may have certain modifications, including nucleotide substitutions, additions or deletions, which
20 all still code for polypeptides having substantially the same activity. The term "DNA sequence encoding a vertebrate *hh* polypeptide" may thus refer to one or more genes within a particular individual. Moreover, certain differences in nucleotide sequences may exist between individual organisms, which are called alleles. Such allelic differences may or may not result in differences in amino acid sequence of the encoded polypeptide yet still encode a
25 protein with the same biological activity.

"Homology" refers to sequence similarity between two peptides or between two nucleic acid molecules. Homology can be determined by comparing a position in each sequence which may be aligned for purposes of comparison. When a position in the compared sequence is occupied by the same base or amino acid, then the molecules are
30 homologous at that position. A degree of homology between sequences is a function of the number of matching or homologous positions shared by the sequences. An "unrelated" or "non-homologous" sequence shares less than 40 percent identity, though preferably less than 25 percent identity, with one of the vertebrate *hh* sequences of the present invention.

"Cells," "host cells" or "recombinant host cells" are terms used interchangeably
35 herein. It is understood that such terms refer not only to the particular subject cell but to the progeny or potential progeny of such a cell. Because certain modifications may occur in

succeeding generations due to either mutation or environmental influences, such progeny may not, in fact, be identical to the parent cell, but are still included within the scope of the term as used herein.

A "chimeric protein" or "fusion protein" is a fusion of a first amino acid sequence encoding one of the subject vertebrate *hh* polypeptides with a second amino acid sequence defining a domain foreign to and not substantially homologous with any domain of one of the vertebrate *hh* proteins. A chimeric protein may present a foreign domain which is found (albeit in a different protein) in an organism which also expresses the first protein, or it may be an "interspecies", "intergenic", etc. fusion of protein structures expressed by different kinds of organisms. In general, a fusion protein can be represented by the general formula X-*hh*-Y, wherein *hh* represents a portion of the protein which is derived from one of the vertebrate *hh* proteins, and X and Y are independently absent or represent amino acid sequences which are not related to one of the vertebrate *hh* sequences in an organism, including naturally occurring mutants.

As used herein, the terms "transforming growth factor-beta" and "TGF- β " denote a family of structurally related paracrine polypeptides found ubiquitously in vertebrates, and prototypic of a large family of metazoan growth, differentiation, and morphogenesis factors (see, for review, Massague et al. (1990) *Ann Rev Cell Biol* 6:597-641; and Sporn et al. (1992) *J Cell Biol* 119:1017-1021). Included in this family are the "bone morphogenetic proteins" or "BMPs", which refers to proteins isolated from bone, and fragments thereof and synthetic peptides which are capable of inducing bone deposition alone or when combined with appropriate cofactors. Preparation of BMPs, such as BMP-1, -2, -3, and -4, is described in, for example, PCT publication WO 88/00205. Wozney (1989) *Growth Fact Res* 1:267-280 describes additional BMP proteins closely related to BMP-2, and which have been designated BMP-5, -6, and -7. PCT publications WO89/09787 and WO89/09788 describe a protein called "OP-1," now known to be BMP-7. Other BMPs are known in the art.

The term "isolated" as also used herein with respect to nucleic acids, such as DNA or RNA, refers to molecules separated from other DNAs, or RNAs, respectively, that are present in the natural source of the macromolecule. For example, an isolated nucleic acid encoding one of the subject vertebrate *hh* polypeptides preferably includes no more than 10 kilobases (kb) of nucleic acid sequence which naturally immediately flanks the vertebrate *hh* gene in genomic DNA, more preferably no more than 5kb of such naturally occurring flanking sequences, and most preferably less than 1.5kb of such naturally occurring flanking sequence. The term isolated as used herein also refers to a nucleic acid or peptide that is substantially free of cellular material, viral material, or culture medium when produced by recombinant DNA techniques, or chemical precursors or other chemicals when chemically synthesized.

20

Moreover, an "isolated nucleic acid" is meant to include nucleic acid fragments which are not naturally occurring as fragments and would not be found in the natural state.

As described below, one aspect of the invention pertains to isolated nucleic acids comprising the nucleotide sequences encoding vertebrate *hh* homologues, and/or equivalents of such nucleic acids. The term nucleic acid as used herein is intended to include fragments as equivalents. The term equivalent is understood to include nucleotide sequences encoding functionally equivalent *hh* polypeptides or functionally equivalent peptides having an activity of a vertebrate *hh* protein such as described herein. Equivalent nucleotide sequences will include sequences that differ by one or more nucleotide substitutions, additions or deletions, such as allelic variants; and will, therefore, include sequences that differ from the nucleotide sequence of the vertebrate *hh* cDNAs shown in SEQ ID Nos:1-7 due to the degeneracy of the genetic code. Equivalents will also include nucleotide sequences that hybridize under stringent conditions (i.e., equivalent to about 20-27°C below the melting temperature (T_m) of the DNA duplex formed in about 1M salt) to the nucleotide sequences represented in SEQ ID Nos:1-7. In one embodiment, equivalents will further include nucleic acid sequences derived from and evolutionarily related to, a nucleotide sequences shown in any of SEQ ID Nos:1-7.

Moreover, it will be generally appreciated that, under certain circumstances, it may be advantageous to provide homologs of one of the subject *hedgehog* polypeptides which function in a limited capacity as one of either an *hh* agonist or an *hh* antagonist, in order to promote or inhibit only a subset of the biological activities of the naturally-occurring form of the protein. Thus, specific biological effects can be elicited by treatment with a homolog of limited function, and with fewer side effects relative to treatment with agonists or antagonists which are directed to all of the biological activities of naturally occurring forms of *hedgehog* proteins.

Homologs of one of the subject *hedgehog* proteins can be generated by mutagenesis, such as by discrete point mutation(s), or by truncation. For instance, mutation can give rise to homologs which retain substantially the same, or merely a subset, of the biological activity of the *hh* polypeptide from which it was derived. Alternatively, antagonistic forms of the protein can be generated which are able to inhibit the function of the naturally occurring form of the protein, such as by competitively binding to an *hh* receptor.

Polypeptides referred to herein as having an activity of a vertebrate *hh* protein are defined as peptides that have an amino acid sequence corresponding to all or a portion of the amino acid sequences of a vertebrate *hh* proteins shown in any of SEQ ID No:8, SEQ ID No:9, SEQ ID No:10, SEQ ID No:11, SEQ ID No:12, SEQ ID No:13 or SEQ ID No:14 and which have at least one biological activity of a vertebrate *hh* protein. Examples of such biological activity of a vertebrate *hh* protein include the ability to induce (or otherwise

21

modulate) formation and differentiation of the head, limbs, lungs, central nervous system (CNS), or mesodermal patterning of developing vertebrate embryos. In preferred embodiments, the biological activity can comprise an ability to regulate neurogenesis, such as a motor neuron inducing activity, a neuronal differentiation inducing activity, or a neuronal survival promoting activity. *Hedgehog* proteins of the present invention can also have biological activities which include an ability to regulate organogenesis, such as through the ability to influence limb patterning, by, for example, skeletogenic activity. The biological activity associated with the *hedgehog* proteins of the present invention can also include the ability to induce stem cell or germ cell differentiation, including the ability to induce differentiation of chondrocytes or an involvement in spermatogenesis. *Hedgehog* proteins of the present invention can also be characterized in terms of biological activities which include: an ability to modulate proliferation, survival and/or differentiation of mesodermally-derived tissue, such as tissue derived from dorsal mesoderm; the ability to modulate proliferation, survival and/or differentiation of ectodermally-derived tissue, such as tissue derived from the neural tube, neural crest, or head mesenchyme; the ability to modulate proliferation, survival and/or differentiation of endodermally-derived tissue, such as tissue derived from the primitive gut. Moreover, as described in the Examples below, the subject *hedgehog* proteins have the ability to induce expression of secondary signaling molecules, such as members of the Transforming Growth Factor β (TGF β) family, including bone morphogenic proteins, e.g. *BMP-2* and *BMP-4*, as well as members of the fibroblast growth factor (FGF) family, such as *Fgf-4*. Other biological activities of the subject *hedgehog* proteins are described herein or will be reasonably apparent to those skilled in the art. According to the present invention, a polypeptide has biological activity if it is a specific agonist or antagonist of a naturally-occurring form of a vertebrate *hedgehog* protein.

Preferred nucleic acids encode a vertebrate *hedgehog* polypeptide comprising an amino acid sequence at least 60% homologous, more preferably 70% homologous and most preferably 80% homologous with an amino acid sequence selected from the group consisting of SEQ ID Nos:8-14. Nucleic acids which encode polypeptides at least about 90%, more preferably at least about 95%, and most preferably at least about 98-99% homology with an amino acid sequence represented in one of SEQ ID Nos:8-14 are also within the scope of the invention. In one embodiment, the nucleic acid is a cDNA encoding a peptide having at least one activity of the subject vertebrate *hh* polypeptide. Preferably, the nucleic acid includes all or a portion of the nucleotide sequence corresponding to the coding region of SEQ ID Nos:1-7.

Another aspect of the invention provides a nucleic acid which hybridizes under high or low stringency conditions to a nucleic acid represented by one of SEQ ID Nos:1-7. Appropriate stringency conditions which promote DNA hybridization, for example, 6.0 x

22

sodium chloride/sodium citrate (SSC) at about 45°C, followed by a wash of 2.0 x SSC at 50°C, are known to those skilled in the art or can be found in *Current Protocols in Molecular Biology*, John Wiley & Sons, N.Y. (1989), 6.3.1-6.3.6. For example, the salt concentration in the wash step can be selected from a low stringency of about 2.0 x SSC at 50°C to a high stringency of about 0.2 x SSC at 50°C. In addition, the temperature in the wash step can be increased from low stringency conditions at room temperature, about 22°C, to high stringency conditions at about 65°C.

Nucleic acids, having a sequence that differs from the nucleotide sequences shown in one of SEQ ID No:1, SEQ ID No:2, SEQ ID No:3, SEQ ID No:4, SEQ ID No:5, SEQ ID No:6 or SEQ ID No:7 due to degeneracy in the genetic code are also within the scope of the invention. Such nucleic acids encode functionally equivalent peptides (i.e., a peptide having a biological activity of a vertebrate *hh* polypeptide) but differ in sequence from the sequence shown in the sequence listing due to degeneracy in the genetic code. For example, a number of amino acids are designated by more than one triplet. Codons that specify the same amino acid, or synonyms (for example, CAU and CAC each encode histidine) may result in "silent" mutations which do not affect the amino acid sequence of a vertebrate *hh* polypeptide. However, it is expected that DNA sequence polymorphisms that do lead to changes in the amino acid sequences of the subject *hh* polypeptides will exist among vertebrates. One skilled in the art will appreciate that these variations in one or more nucleotides (up to about 3-5% of the nucleotides) of the nucleic acids encoding polypeptides having an activity of a vertebrate *hh* polypeptide may exist among individuals of a given species due to natural allelic variation.

Fragments of the nucleic acids encoding an active portion of the vertebrate *hedgehog* proteins are also within the scope of the invention. As used herein, a *hedgehog* gene fragment refers to a nucleic acid having fewer nucleotides than the nucleotide sequence encoding the entire amino acid sequence of a vertebrate *hh* protein represented in SEQ ID No:8, SEQ ID No:9, SEQ ID No:10, SEQ ID No:11, SEQ ID No:12, SEQ ID No:13 or SEQ ID No:14, yet which (preferably) encodes a peptide which retains some biological activity of the full length protein, e.g. the fragment retains the ability to induce formation and differentiation of the head, limbs, lungs, central nervous system (CNS), or mesodermal patterning of developing vertebrate embryo. Nucleic acid fragments within the scope of the present invention include those capable of hybridizing under high or low stringency conditions with nucleic acids from other species for use in screening protocols to detect other *hedgehog* homologs, as well as those capable of hybridizing with nucleic acids from human specimens for use in detecting the presence of a nucleic acid encoding a *hedgehog* protein, including alternate isoforms, e.g. mRNA splicing variants. Nucleic acids within the scope of the invention may also contain linker sequences, modified restriction endonuclease sites and

other sequences useful for molecular cloning,²³ expression or purification of recombinant forms of the subject *hh* polypeptides.

As indicated by the examples set out below, *hedgehog* protein-encoding nucleic acids can be obtained from mRNA present in any of a number of eukaryotic cells. It should also be possible to obtain nucleic acids encoding vertebrate *hh* polypeptides of the present invention from genomic DNA obtained from both adults and embryos. For example, a gene encoding a *hh* protein can be cloned from either a cDNA or a genomic library in accordance with protocols described herein, as well as those generally known to persons skilled in the art. A cDNA encoding a *hedgehog* protein can be obtained by isolating total mRNA from a cell, e.g. a mammalian cell, e.g. a human cell, including embryonic cells. Double stranded cDNAs can then be prepared from the total mRNA, and subsequently inserted into a suitable plasmid or bacteriophage vector using any one of a number of known techniques. The gene encoding a vertebrate *hh* protein can also be cloned using established polymerase chain reaction techniques in accordance with the nucleotide sequence information provided by the invention. The nucleic acid of the invention can be DNA or RNA. A preferred nucleic acid is a cDNA represented by a sequence selected from the group consisting of SEQ ID Nos:1-7.

Another aspect of the invention relates to the use of the isolated nucleic acid in "antisense" therapy. As used herein, "antisense" therapy refers to administration or *in situ* generation of oligonucleotide probes or their derivatives which specifically hybridizes (e.g. binds) under cellular conditions, with the cellular mRNA and/or genomic DNA encoding one or more of the subject *hedgehog* proteins so as to inhibit expression of that protein, e.g. by inhibiting transcription and/or translation. The binding may be by conventional base pair complementarity, or, for example, in the case of binding to DNA duplexes, through specific interactions in the major groove of the double helix. In general, "antisense" therapy refers to the range of techniques generally employed in the art, and includes any therapy which relies on specific binding to oligonucleotide sequences.

An antisense construct of the present invention can be delivered, for example, as an expression plasmid which, when transcribed in the cell, produces RNA which is complementary to at least a unique portion of the cellular mRNA which encodes a vertebrate *hh* protein. Alternatively, the antisense construct is an oligonucleotide probe which is generated *ex vivo* and which, when introduced into the cell causes inhibition of expression by hybridizing with the mRNA and/or genomic sequences of a vertebrate *hh* gene. Such oligonucleotide probes are preferably modified oligonucleotide which are resistant to endogenous nucleases, e.g. exonucleases and/or endonucleases, and is therefore stable *in vivo*. Exemplary nucleic acid molecules for use as antisense oligonucleotides are phosphoramidate, phosphothioate and methylphosphonate analogs of DNA (see also U.S. Patents 5,176,996; 5,264,564; and 5,256,775). Additionally, general approaches to

24

constructing oligomers useful in antisense therapy have been reviewed, for example, by Van der Krol et al. (1988) *Biotechniques* 6:958-976; and Stein et al. (1988) *Cancer Res* 48:2659-2668.

Accordingly, the modified oligomers of the invention are useful in therapeutic, diagnostic, and research contexts. In therapeutic applications, the oligomers are utilized in a manner appropriate for antisense therapy in general. For such therapy, the oligomers of the invention can be formulated for a variety of loads of administration, including systemic and topical or localized administration. Techniques and formulations generally may be found in Remington's Pharmaceutical Sciences, Meade Publishing Co., Easton, PA. For systemic administration, injection is preferred, including intramuscular, intravenous, intraperitoneal, and subcutaneous for injection, the oligomers of the invention can be formulated in liquid solutions, preferably in physiologically compatible buffers such as Hank's solution or Ringer's solution. In addition, the oligomers may be formulated in solid form and redissolved or suspended immediately prior to use. Lyophilized forms are also included.

Systemic administration can also be by transmucosal or transdermal means, or the compounds can be administered orally. For transmucosal or transdermal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art, and include, for example, for transmucosal administration bile salts and fusidic acid derivatives. In addition, detergents may be used to facilitate permeation. Transmucosal administration may be through nasal sprays or using suppositories. For oral administration, the oligomers are formulated into conventional oral administration forms such as capsules, tablets, and tonics. For topical administration, the oligomers of the invention are formulated into ointments, salves, gels, or creams as generally known in the art.

In addition to use in therapy, the oligomers of the invention may be used as diagnostic reagents to detect the presence or absence of the target DNA or RNA sequences to which they specifically bind. Such diagnostic tests are described in further detail below.

Likewise, the antisense constructs of the present invention, by antagonizing the normal biological activity of one of the *hedgehog* proteins, can be used in the manipulation of tissue, e.g. tissue differentiation, both *in vivo* and in *ex vivo* tissue cultures.

Also, the anti-sense techniques (e.g. microinjection of antisense molecules, or transfection with plasmids whose transcripts are anti-sense with regard to an *hh* mRNA or gene sequence) can be used to investigate role of *hh* in developmental events, as well as the normal cellular function of *hh* in adult tissue. Such techniques can be utilized in cell culture, but can also be used in the creation of transgenic animals.

25

This invention also provides expression vectors containing a nucleic acid encoding a vertebrate *hh* polypeptide, operably linked to at least one transcriptional regulatory sequence. Operably linked is intended to mean that the nucleotide sequence is linked to a regulatory sequence in a manner which allows expression of the nucleotide sequence. Regulatory sequences are art-recognized and are selected to direct expression of the subject vertebrate *hh* proteins. Accordingly, the term transcriptional regulatory sequence includes promoters, enhancers and other expression control elements. Such regulatory sequences are described in Goeddel; *Gene Expression Technology: Methods in Enzymology* 185, Academic Press, San Diego, CA (1990). For instance, any of a wide variety of expression control sequences, sequences that control the expression of a DNA sequence when operatively linked to it, may be used in these vectors to express DNA sequences encoding vertebrate *hh* polypeptides of this invention. Such useful expression control sequences, include, for example, a viral LTR, such as the LTR of the Moloney murine leukemia virus, the early and late promoters of SV40, adenovirus or cytomegalovirus immediate early promoter, the lac system, the trp system, the TAC or TRC system, T7 promoter whose expression is directed by T7 RNA polymerase, the major operator and promoter regions of phage λ , the control regions for fd coat protein, the promoter for 3-phosphoglycerate kinase or other glycolytic enzymes, the promoters of acid phosphatase, e.g., Pho5, the promoters of the yeast α -mating factors, the polyhedron promoter of the baculovirus system and other sequences known to control the expression of genes of prokaryotic or eukaryotic cells or their viruses, and various combinations thereof. It should be understood that the design of the expression vector may depend on such factors as the choice of the host cell to be transformed and/or the type of protein desired to be expressed. Moreover, the vector's copy number, the ability to control that copy number and the expression of any other proteins encoded by the vector, such as antibiotic markers, should also be considered. In one embodiment, the expression vector includes a recombinant gene encoding a peptide having an agonistic activity of a subject *hedgehog* polypeptide, or alternatively, encoding a peptide which is an antagonistic form of the *hh* protein. Such expression vectors can be used to transfect cells and thereby produce polypeptides, including fusion proteins, encoded by nucleic acids as described herein.

Moreover, the gene constructs of the present invention can also be used as a part of a gene therapy protocol to deliver nucleic acids encoding either an agonistic or antagonistic form of one of the subject vertebrate *hedgehog* proteins. Thus, another aspect of the invention features expression vectors for *in vivo* or *in vitro* transfection and expression of a vertebrate *hh* polypeptide in particular cell types so as to reconstitute the function of, or alternatively, abrogate the function of *hedgehog*-induced signaling in a tissue in which the naturally-occurring form of the protein is misexpressed; or to deliver a form of the protein which alters differentiation of tissue, or which inhibits neoplastic transformation.

26

Expression constructs of the subject vertebrate *hh* polypeptide, and mutants thereof, may be administered in any biologically effective carrier, e.g. any formulation or composition capable of effectively delivering the recombinant gene to cells *in vivo*. Approaches include insertion of the subject gene in viral vectors including recombinant retroviruses, adenovirus, adeno-associated virus, and herpes simplex virus-1, or recombinant bacterial or eukaryotic plasmids. Viral vectors transfect cells directly; plasmid DNA can be delivered with the help of, for example, cationic liposomes (lipofectin) or derivatized (e.g. antibody conjugated), polylysine conjugates, gramicidin S, artificial viral envelopes or other such intracellular carriers, as well as direct injection of the gene construct or CaPO_4 precipitation carried out *in vivo*. It will be appreciated that because transduction of appropriate target cells represents the critical first step in gene therapy, choice of the particular gene delivery system will depend on such factors as the phenotype of the intended target and the route of administration, e.g. locally or systemically. Furthermore, it will be recognized that the particular gene construct provided for *in vivo* transduction of *hedgehog* expression are also useful for *in vitro* transduction of cells, such as for use in the *ex vivo* tissue culture systems described below.

A preferred approach for *in vivo* introduction of nucleic acid into a cell is by use of a viral vector containing nucleic acid, e.g. a cDNA, encoding the particular form of the *hedgehog* polypeptide desired. Infection of cells with a viral vector has the advantage that a large proportion of the targeted cells can receive the nucleic acid. Additionally, molecules encoded within the viral vector, e.g., by a cDNA contained in the viral vector, are expressed efficiently in cells which have taken up viral vector nucleic acid.

Retrovirus vectors and adeno-associated virus vectors are generally understood to be the recombinant gene delivery system of choice for the transfer of exogenous genes *in vivo*, particularly into humans. These vectors provide efficient delivery of genes into cells, and the transferred nucleic acids are stably integrated into the chromosomal DNA of the host. A major prerequisite for the use of retroviruses is to ensure the safety of their use, particularly with regard to the possibility of the spread of wild-type virus in the cell population. The development of specialized cell lines (termed "packaging cells") which produce only replication-defective retroviruses has increased the utility of retroviruses for gene therapy, and defective retroviruses are well characterized for use in gene transfer for gene therapy purposes (for a review see Miller, A.D. (1990) *Blood* 76:271). Thus, recombinant retrovirus can be constructed in which part of the retroviral coding sequence (*gag*, *pol*, *env*) has been replaced by nucleic acid encoding one of the subject proteins rendering the retrovirus replication defective. The replication defective retrovirus is then packaged into virions which can be used to infect a target cell through the use of a helper virus by standard techniques. Protocols for producing recombinant retroviruses and for infecting cells *in vitro* or *in vivo*

with such viruses can be found in Current Protocols in Molecular Biology, Ausubel, F.M. et al. (eds.) Greene Publishing Associates, (1989), Sections 9.10-9.14 and other standard laboratory manuals. Examples of suitable retroviruses include pLJ, pZIP, pWE and pEM which are well known to those skilled in the art. Examples of suitable packaging virus lines for preparing both ecotropic and amphotropic retroviral systems include ψ Crip, ψ Cre, ψ 2 and ψ Am. Retroviruses have been used to introduce a variety of genes into many different cell types, including neuronal cells, *in vitro* and/or *in vivo* (see for example Eglitis, et al. (1985) *Science* 230:1395-1398; Danos and Mulligan (1988) *Proc. Natl. Acad. Sci. USA* 85:6460-6464; Wilson et al. (1988) *Proc. Natl. Acad. Sci. USA* 85:3014-3018; Armentano et al. (1990) *Proc. Natl. Acad. Sci. USA* 87:6141-6145; Huber et al. (1991) *Proc. Natl. Acad. Sci. USA* 88:8039-8043; Ferry et al. (1991) *Proc. Natl. Acad. Sci. USA* 88:8377-8381; Chowdhury et al. (1991) *Science* 254:1802-1805; van Beusechem et al. (1992) *Proc. Natl. Acad. Sci. USA* 89:7640-7644; Kay et al. (1992) *Human Gene Therapy* 3:641-647; Dai et al. (1992) *Proc. Natl. Acad. Sci. USA* 89:10892-10895; Hwu et al. (1993) *J. Immunol.* 150:4104-4115; U.S. Patent No. 4,868,116; U.S. Patent No. 4,980,286; PCT Application WO 89/07136; PCT Application WO 89/02468; PCT Application WO 89/05345; and PCT Application WO 92/07573).

Furthermore, it has been shown that it is possible to limit the infection spectrum of retroviruses and consequently of retroviral-based vectors, by modifying the viral packaging proteins on the surface of the viral particle (see, for example PCT publications WO93/25234 and WO94/06920). For instance, strategies for the modification of the infection spectrum of retroviral vectors include: coupling antibodies specific for cell surface antigens to the viral *env* protein (Roux et al. (1989) *PNAS* 86:9079-9083; Julian et al. (1992) *J. Gen Virol* 73:3251-3255; and Goud et al. (1983) *Virology* 163:251-254); or coupling cell surface receptor ligands to the viral *env* proteins (Neda et al. (1991) *J Biol Chem* 266:14143-14146). Coupling can be in the form of the chemical cross-linking with a protein or other variety (e.g. lactose to convert the *env* protein to an asialoglycoprotein), as well as by generating fusion proteins (e.g. single-chain antibody/*env* fusion proteins). This technique, while useful to limit or otherwise direct the infection to certain tissue types, can also be used to convert an ecotropic vector in to an amphotropic vector.

Moreover, use of retroviral gene delivery can be further enhanced by the use of tissue- or cell-specific transcriptional regulatory sequences which control expression of the *hh* gene of the retroviral vector.

Another viral gene delivery system useful in the present invention utilizes adenovirus-derived vectors. The genome of an adenovirus can be manipulated such that it encodes and expresses a gene product of interest but is inactivated in terms of its ability to replicate in a normal lytic viral life cycle. See for example Berkner et al. (1988) *BioTechniques* 6:616;

Rosenfeld et al. (1991) *Science* 252:431-434; and Rosenfeld et al. (1992) *Cell* 68:143-155. Suitable adenoviral vectors derived from the adenovirus strain Ad type 5 dl324 or other strains of adenovirus (e.g., Ad2, Ad3, Ad7 etc.) are well known to those skilled in the art. Recombinant adenoviruses can be advantageous in certain circumstances in that they can be used to infect a wide variety of cell types, including airway epithelium (Rosenfeld et al. (1992) cited *supra*), endothelial cells (Lemarchand et al. (1992) *Proc. Natl. Acad. Sci. USA* 89:6482-6486), hepatocytes (Herz and Gerard (1993) *Proc. Natl. Acad. Sci. USA* 90:2812-2816) and muscle cells (Quantin et al. (1992) *Proc. Natl. Acad. Sci. USA* 89:2581-2584). Furthermore, the virus particle is relatively stable and amenable to purification and concentration, and as above, can be modified so as to affect the spectrum of infectivity. Additionally, introduced adenoviral DNA (and foreign DNA contained therein) is not integrated into the genome of a host cell but remains episomal, thereby avoiding potential problems that can occur as a result of insertional mutagenesis in situations where introduced DNA becomes integrated into the host genome (e.g., retroviral DNA). Moreover, the carrying capacity of the adenoviral genome for foreign DNA is large (up to 8 kilobases) relative to other gene delivery vectors (Berkner et al. cited *supra*; Haj-Ahmand and Graham (1986) *J. Virol.* 57:267). Most replication-defective adenoviral vectors currently in use and therefore favored by the present invention are deleted for all or parts of the viral E1 and E3 genes but retain as much as 80% of the adenoviral genetic material (see, e.g., Jones et al. (1979) *Cell* 16:683; Berkner et al., *supra*; and Graham et al. in Methods in Molecular Biology, E.J. Murray, Ed. (Humana, Clifton, NJ, 1991) vol. 7. pp. 109-127). Expression of the inserted *hedgehog* gene can be under control of, for example, the E1A promoter, the major late promoter (MLP) and associated leader sequences, the E3 promoter, or exogenously added promoter sequences.

Yet another viral vector system useful for delivery of one of the subject vertebrate *hh* genes is the adeno-associated virus (AAV). Adeno-associated virus is a naturally occurring defective virus that requires another virus, such as an adenovirus or a herpes virus, as a helper virus for efficient replication and a productive life cycle. (For a review see Muzyczka et al. *Curr. Topics in Micro. and Immunol.* (1992) 158:97-129). It is also one of the few viruses that may integrate its DNA into non-dividing cells, and exhibits a high frequency of stable integration (see for example Flotte et al. (1992) *Am. J. Respir. Cell. Mol. Biol.* 7:349-356; Samulski et al. (1989) *J. Virol.* 63:3822-3828; and McLaughlin et al. (1989) *J. Virol.* 62:1963-1973). Vectors containing as little as 300 base pairs of AAV can be packaged and can integrate. Space for exogenous DNA is limited to about 4.5 kb. An AAV vector such as that described in Tratschin et al. (1985) *Mol. Cell. Biol.* 5:3251-3260 can be used to introduce DNA into cells. A variety of nucleic acids have been introduced into different cell types using AAV vectors (see for example Hermonat et al. (1984) *Proc. Natl. Acad. Sci. USA* 81:6466-6470; Tratschin et al. (1985) *Mol. Cell. Biol.* 4:2072-2081; Wondisford et al. (1988)

Mol. Endocrinol. 2:32-39; Tratschin et al. (1984) *J. Virol.* 51:611-619; and Flotte et al. (1993) *J. Biol. Chem.* 268:3781-3790).

In addition to viral transfer methods, such as those illustrated above, non-viral methods can also be employed to cause expression of a subject *hedgehog* polypeptide in the tissue of an animal. Most nonviral methods of gene transfer rely on normal mechanisms used by mammalian cells for the uptake and intracellular transport of macromolecules. In preferred embodiments, non-viral gene delivery systems of the present invention rely on endocytic pathways for the uptake of the subject *hh* polypeptide gene by the targeted cell. Exemplary gene delivery systems of this type include liposomal derived systems, poly-lysine conjugates, and artificial viral envelopes.

In clinical settings, the gene delivery systems for the therapeutic *hedgehog* gene can be introduced into a patient by any of a number of methods, each of which is familiar in the art. For instance, a pharmaceutical preparation of the gene delivery system can be introduced systemically, e.g. by intravenous injection, and specific transduction of the protein in the target cells occurs predominantly from specificity of transfection provided by the gene delivery vehicle, cell-type or tissue-type expression due to the transcriptional regulatory sequences controlling expression of the receptor gene, or a combination thereof. In other embodiments, initial delivery of the recombinant gene is more limited with introduction into the animal being quite localized. For example, the gene delivery vehicle can be introduced by catheter (see U.S. Patent 5,328,470) or by stereotactic injection (e.g. Chen et al. (1994) *PNAS* 91: 3054-3057). A vertebrate *hh* gene, such as any one of the clones represented in the group consisting of SEQ ID NO:1-7, can be delivered in a gene therapy construct by electroporation using techniques described, for example, by Dev et al. ((1994) *Cancer Treat Rev* 20:105-115).

The pharmaceutical preparation of the gene therapy construct can consist essentially of the gene delivery system in an acceptable diluent, or can comprise a slow release matrix in which the gene delivery vehicle is imbedded. Alternatively, where the complete gene delivery system can be produced intact from recombinant cells, e.g. retroviral vectors, the pharmaceutical preparation can comprise one or more cells which produce the gene delivery system.

Another aspect of the present invention concerns recombinant forms of the *hedgehog* proteins. Recombinant polypeptides preferred by the present invention, in addition to native *hedgehog* proteins, are at least 60% homologous, more preferably 70% homologous and most preferably 80% homologous with an amino acid sequence represented by any of SEQ ID Nos:8-14. Polypeptides which possess an activity of a *hedgehog* protein (i.e. either agonistic or antagonistic), and which are at least 90%, more preferably at least 95%, and most

preferably at least about 98-99% homologous³⁰ with a sequence selected from the group consisting of SEQ ID Nos:8-14 are also within the scope of the invention.

The term "recombinant protein" refers to a polypeptide of the present invention which is produced by recombinant DNA techniques, wherein generally, DNA encoding a vertebrate *hh* polypeptide is inserted into a suitable expression vector which is in turn used to transform a host cell to produce the heterologous protein. Moreover, the phrase "derived from", with respect to a recombinant *hedgehog* gene, is meant to include within the meaning of "recombinant protein" those proteins having an amino acid sequence of a native *hedgehog* protein, or an amino acid sequence similar thereto which is generated by mutations including substitutions and deletions (including truncation) of a naturally occurring form of the protein.

The present invention further pertains to recombinant forms of one of the subject *hedgehog* polypeptides which are encoded by genes derived from a vertebrate organism, particularly a mammal (e.g. a human), and which have amino acid sequences evolutionarily related to the *hedgehog* proteins represented in SEQ ID Nos:8-14. Such recombinant *hh* polypeptides preferably are capable of functioning in one of either role of an agonist or antagonist of at least one biological activity of a wild-type ("authentic") *hedgehog* protein of the appended sequence listing. The term "evolutionarily related to", with respect to amino acid sequences of vertebrate *hedgehog* proteins, refers to both polypeptides having amino acid sequences which have arisen naturally, and also to mutational variants of vertebrate *hh* polypeptides which are derived, for example, by combinatorial mutagenesis. Such evolutionarily derived *hedgehog* proteins polypeptides preferred by the present invention are at least 60% homologous, more preferably 70% homologous and most preferably 80% homologous with the amino acid sequence selected from the group consisting of SEQ ID Nos:8-14. Polypeptides having at least about 90%, more preferably at least about 95%, and most preferably at least about 98-99% homology with a sequence selected from the group consisting of SEQ ID Nos:8-14 are also within the scope of the invention.

The present invention further pertains to methods of producing the subject *hedgehog* polypeptides. For example, a host cell transfected with a nucleic acid vector directing expression of a nucleotide sequence encoding the subject polypeptides can be cultured under appropriate conditions to allow expression of the peptide to occur. The polypeptide *hedgehog* may be secreted and isolated from a mixture of cells and medium containing the recombinant vertebrate *hh* polypeptide. Alternatively, the peptide may be retained cytoplasmically by removing the signal peptide sequence from the recombinant *hh* gene and the cells harvested, lysed and the protein isolated. A cell culture includes host cells, media and other byproducts. Suitable media for cell culture are well known in the art. The recombinant *hh* polypeptide can be isolated from cell culture medium, host cells, or both using techniques known in the art for purifying proteins including ion-exchange

31

chromatography, gel filtration chromatography, ultrafiltration, electrophoresis, and immunoaffinity purification with antibodies specific for such peptide. In a preferred embodiment, the recombinant *hh* polypeptide is a fusion protein containing a domain which facilitates its purification, such as an *hh*/GST fusion protein.

5 This invention also pertains to a host cell transfected to express a recombinant form of the subject *hedgehog* polypeptides. The host cell may be any prokaryotic or eukaryotic cell. Thus, a nucleotide sequence derived from the cloning of vertebrate *hedgehog* proteins, encoding all or a selected portion of the full-length protein, can be used to produce a recombinant form of a vertebrate *hh* polypeptide via microbial or eukaryotic cellular
10 processes. Ligating the polynucleotide sequence into a gene construct, such as an expression vector, and transforming or transfecting into hosts, either eukaryotic (yeast, avian, insect or mammalian) or prokaryotic (bacterial cells), are standard procedures used in producing other well-known proteins, e.g. insulin, interferons, human growth hormone, IL-1, IL-2, and the like. Similar procedures, or modifications thereof, can be employed to prepare recombinant
15 *hedgehog* polypeptides by microbial means or tissue-culture technology in accord with the subject invention.

The recombinant *hedgehog* genes can be produced by ligating nucleic acid encoding an *hh* protein, or a portion thereof, into a vector suitable for expression in either prokaryotic cells, eukaryotic cells, or both. Expression vectors for production of recombinant forms of
20 the subject *hh* polypeptides include plasmids and other vectors. For instance, suitable vectors for the expression of a *hedgehog* polypeptide include plasmids of the types: pBR322-derived plasmids, pEMBL-derived plasmids, pEX-derived plasmids, pBTac-derived plasmids and pUC-derived plasmids for expression in prokaryotic cells, such as *E. coli*.

A number of vectors exist for the expression of recombinant proteins in yeast. For
25 instance, YEP24, YIP5, YEP51, YEP52, pYES2, and YRP17 are cloning and expression vehicles useful in the introduction of genetic constructs into *S. cerevisiae* (see, for example, Broach *et al.* (1983) in *Experimental Manipulation of Gene Expression*, ed. M. Inouye Academic Press, p. 83, incorporated by reference herein). These vectors can replicate in *E. coli* due the presence of the pBR322 ori, and in *S. cerevisiae* due to the replication
30 determinant of the yeast 2 micron plasmid. In addition, drug resistance markers such as ampicillin can be used. In an illustrative embodiment, an *hh* polypeptide is produced recombinantly utilizing an expression vector generated by sub-cloning the coding sequence of one of the *hedgehog* genes represented in SEQ ID Nos:1-7.

The preferred mammalian expression vectors contain both prokaryotic sequences, to
35 facilitate the propagation of the vector in bacteria, and one or more eukaryotic transcription units that are expressed in eukaryotic cells. The pcDNAI/amp, pcDNAI/neo, pRc/CMV,

pSV2gpt, pSV2neo, pSV2-dhfr, pTk2, pRSVneo, pMSG, pSVT7, pko-neo and pHyg derived vectors are examples of mammalian expression vectors suitable for transfection of eukaryotic cells. Some of these vectors are modified with sequences from bacterial plasmids, such as pBR322, to facilitate replication and drug resistance selection in both prokaryotic and eukaryotic cells. Alternatively, derivatives of viruses such as the bovine papillomavirus (BPV-1), or Epstein-Barr virus (pHEBo, pREP-derived and p205) can be used for transient expression of proteins in eukaryotic cells. The various methods employed in the preparation of the plasmids and transformation of host organisms are well known in the art. For other suitable expression systems for both prokaryotic and eukaryotic cells, as well as general recombinant procedures, see *Molecular Cloning A Laboratory Manual*, 2nd Ed., ed. by Sambrook, Fritsch and Maniatis (Cold Spring Harbor Laboratory Press: 1989) Chapters 16 and 17.

In some instances, it may be desirable to express the recombinant *hedgehog* polypeptide by the use of a baculovirus expression system. Examples of such baculovirus expression systems include pVL-derived vectors (such as pVL1392, pVL1393 and pVL941), pAcUW-derived vectors (such as pAcUW1), and pBlueBac-derived vectors (such as the β -gal containing pBlueBac III).

When it is desirable to express only a portion of an *hh* protein, such as a form lacking a portion of the N-terminus, i.e. a truncation mutant which lacks the signal peptide, it may be necessary to add a start codon (ATG) to the oligonucleotide fragment containing the desired sequence to be expressed. It is well known in the art that a methionine at the N-terminal position can be enzymatically cleaved by the use of the enzyme methionine aminopeptidase (MAP). MAP has been cloned from *E. coli* (Ben-Bassat et al. (1987) *J. Bacteriol.* 169:751-757) and *Salmonella typhimurium* and its *in vitro* activity has been demonstrated on recombinant proteins (Miller et al. (1987) *PNAS* 84:2718-1722). Therefore, removal of an N-terminal methionine, if desired, can be achieved either *in vivo* by expressing *hedgehog*-derived polypeptides in a host which produces MAP (e.g., *E. coli* or CM89 or *S. cerevisiae*), or *in vitro* by use of purified MAP (e.g., procedure of Miller et al., *supra*).

Alternatively, the coding sequences for the polypeptide can be incorporated as a part of a fusion gene including a nucleotide sequence encoding a different polypeptide. This type of expression system can be useful under conditions where it is desirable to produce an immunogenic fragment of a *hedgehog* protein. For example, the VP6 capsid protein of rotavirus can be used as an immunologic carrier protein for portions of the *hh* polypeptide, either in the monomeric form or in the form of a viral particle. The nucleic acid sequences corresponding to the portion of a subject *hedgehog* protein to which antibodies are to be raised can be incorporated into a fusion gene construct which includes coding sequences for a late vaccinia virus structural protein to produce a set of recombinant viruses expressing

fusion proteins comprising *hh* epitopes as part of the virion. It has been demonstrated with the use of immunogenic fusion proteins utilizing the Hepatitis B surface antigen fusion proteins that recombinant Hepatitis B virions can be utilized in this role as well. Similarly, chimeric constructs coding for fusion proteins containing a portion of an *hh* protein and the poliovirus capsid protein can be created to enhance immunogenicity of the set of polypeptide antigens (see, for example, EP Publication No: 0259149; and Evans et al. (1989) *Nature* 339:385; Huang et al. (1988) *J. Virol.* 62:3855; and Schlienger et al. (1992) *J. Virol.* 66:2).

The Multiple Antigen Peptide system for peptide-based immunization can also be utilized to generate an immunogen, wherein a desired portion of an *hh* polypeptide is obtained directly from organo-chemical synthesis of the peptide onto an oligomeric branching lysine core (see, for example, Posnett et al. (1988) *JBC* 263:1719 and Nardelli et al. (1992) *J. Immunol.* 148:914). Antigenic determinants of *hh* proteins can also be expressed and presented by bacterial cells.

In addition to utilizing fusion proteins to enhance immunogenicity, it is widely appreciated that fusion proteins can also facilitate the expression of proteins, and accordingly, can be used in the expression of the vertebrate *hh* polypeptides of the present invention. For example, *hedgehog* polypeptides can be generated as glutathione-S-transferase (GST-fusion) proteins. Such GST-fusion proteins can enable easy purification of the *hedgehog* polypeptide, as for example by the use of glutathione-derivatized matrices (see, for example, *Current Protocols in Molecular Biology*, eds. Ausubel et al. (N.Y.: John Wiley & Sons, 1991)). In another embodiment, a fusion gene coding for a purification leader sequence, such as a poly-(His)/enterokinase cleavage site sequence, can be used to replace the signal sequence which naturally occurs at the N-terminus of the *hh* protein (e.g., of the pro-form, in order to permit purification of the poly(His)-*hh* protein by affinity chromatography using a Ni²⁺ metal resin. The purification leader sequence can then be subsequently removed by treatment with enterokinase (e.g., see Hochuli et al. (1987) *J. Chromatography* 411:177; and Janknecht et al. *PNAS* 88:8972).

Techniques for making fusion genes are known to those skilled in the art. Essentially, the joining of various DNA fragments coding for different polypeptide sequences is performed in accordance with conventional techniques, employing blunt-ended or stagger-ended termini for ligation, restriction enzyme digestion to provide for appropriate termini, filling-in of cohesive ends as appropriate, alkaline phosphatase treatment to avoid undesirable joining, and enzymatic ligation. In another embodiment, the fusion gene can be synthesized by conventional techniques including automated DNA synthesizers. Alternatively, PCR amplification of gene fragments can be carried out using anchor primers which give rise to complementary overhangs between two consecutive gene fragments which can subsequently

34

be annealed to generate a chimeric gene sequence (see, for example, *Current Protocols in Molecular Biology*, eds. Ausubel et al. John Wiley & Sons: 1992).

Hedgehog polypeptides may also be chemically modified to create *hh* derivatives by forming covalent or aggregate conjugates with other chemical moieties, such as glycosyl groups, lipids, phosphate, acetyl groups and the like. Covalent derivatives of *hedgehog* proteins can be prepared by linking the chemical moieties to functional groups on amino acid sidechains of the protein or at the N-terminus or at the C-terminus of the polypeptide.

For instance, *hedgehog* proteins can be generated to include a moiety, other than sequence naturally associated with the protein, that binds a component of the extracellular matrix and enhances localization of the analog to cell surfaces. For example, sequences derived from the fibronectin "type-III repeat", such as a tetrapeptide sequence R-G-D-S (Pierschbacher et al. (1984) *Nature* 309:30-3; and Kornblihtt et al. (1985) *EMBO* 4:1755-9) can be added to the *hh* polypeptide to support attachment of the chimeric molecule to a cell through binding ECM components (Ruoslahti et al. (1987) *Science* 238:491-497; Pierschbacher et al. (1987) *J. Biol. Chem.* 262:17294-8.; Hynes (1987) *Cell* 48:549-54; and Hynes (1992) *Cell* 69:11-25).

The present invention also makes available isolated *hedgehog* polypeptides which are isolated from, or otherwise substantially free of other cellular and extracellular proteins, especially morphogenic proteins or other extracellular or cell surface associated proteins which may normally be associated with the *hedgehog* polypeptide. The term "substantially free of other cellular or extracellular proteins" (also referred to herein as "contaminating proteins") or "substantially pure or purified preparations" are defined as encompassing preparations of *hh* polypeptides having less than 20% (by dry weight) contaminating protein, and preferably having less than 5% contaminating protein. Functional forms of the subject polypeptides can be prepared, for the first time, as purified preparations by using a cloned gene as described herein. By "purified", it is meant, when referring to a peptide or DNA or RNA sequence, that the indicated molecule is present in the substantial absence of other biological macromolecules, such as other proteins. The term "purified" as used herein preferably means at least 80% by dry weight, more preferably in the range of 95-99% by weight, and most preferably at least 99.8% by weight, of biological macromolecules of the same type present (but water, buffers, and other small molecules, especially molecules having a molecular weight of less than 5000, can be present). The term "pure" as used herein preferably has the same numerical limits as "purified" immediately above. "Isolated" and "purified" do not encompass either natural materials in their native state or natural materials that have been separated into components (e.g., in an acrylamide gel) but not obtained either as pure (e.g. lacking contaminating proteins, or chromatography reagents such as denaturing agents and polymers, e.g. acrylamide or agarose) substances or solutions. In preferred

embodiments, purified *hedgehog* preparations will lack any contaminating proteins from the same animal from that *hedgehog* is normally produced, as can be accomplished by recombinant expression of, for example, a human *hedgehog* protein in a non-human cell.

As described above for recombinant polypeptides, isolated *hh* polypeptides can include all or a portion of the amino acid sequences represented in SEQ ID No:8, SEQ ID No:9, SEQ ID No:10, SEQ ID No:11, SEQ ID No:12, SEQ ID No:13 or SEQ ID No:14, or a homologous sequence thereto. Preferred fragments of the subject *hedgehog* proteins correspond to the N-terminal and C-terminal proteolytic fragments of the mature protein (see, for instance, Examples 6 and 9).

Isolated peptidyl portions of *hedgehog* proteins can be obtained by screening peptides recombinantly produced from the corresponding fragment of the nucleic acid encoding such peptides. In addition, fragments can be chemically synthesized using techniques known in the art such as conventional Merrifield solid phase f-Moc or t-Boc chemistry. For example, a *hedgehog* polypeptide of the present invention may be arbitrarily divided into fragments of desired length with no overlap of the fragments, or preferably divided into overlapping fragments of a desired length. The fragments can be produced (recombinantly or by chemical synthesis) and tested to identify those peptidyl fragments which can function as either agonists or antagonists of a wild-type (e.g., "authentic") *hedgehog* protein.

The recombinant *hedgehog* polypeptides of the present invention also include homologs of the authentic *hedgehog* proteins, such as versions of those protein which are resistant to proteolytic cleavage, as for example, due to mutations which alter potential cleavage sequences or which inactivate an enzymatic activity associated with the protein. *Hedgehog* homologs of the present invention also include proteins which have been post-translationally modified in a manner different than the authentic protein. Exemplary derivatives of vertebrate *hedgehog* proteins include polypeptides which lack N-glycosylation sites (e.g. to produce an unglycosylated protein), or which lack N-terminal and/or C-terminal sequences.

Modification of the structure of the subject vertebrate *hh* polypeptides can be for such purposes as enhancing therapeutic or prophylactic efficacy, or stability (e.g., *ex vivo* shelf life and resistance to proteolytic degradation *in vivo*). Such modified peptides, when designed to retain at least one activity of the naturally-occurring form of the protein, are considered functional equivalents of the *hedgehog* polypeptides described in more detail herein. Such modified peptides can be produced, for instance, by amino acid substitution, deletion, or addition.

For example, it is reasonable to expect that an isolated replacement of a leucine with an isoleucine or valine, an aspartate with a glutamate, a threonine with a serine, or a similar

36

replacement of an amino acid with a structurally related amino acid (i.e. isosteric and/or isoelectric mutations) will not have a major effect on the biological activity of the resulting molecule. Conservative replacements are those that take place within a family of amino acids that are related in their side chains. Genetically encoded amino acids can be divided into four families: (1) acidic = aspartate, glutamate; (2) basic = lysine, arginine, histidine; (3) nonpolar = alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan; and (4) uncharged polar = glycine, asparagine, glutamine, cysteine, serine, threonine, tyrosine. Phenylalanine, tryptophan, and tyrosine are sometimes classified jointly as aromatic amino acids. In similar fashion, the amino acid repertoire can be grouped as (1) acidic = aspartate, glutamate; (2) basic = lysine, arginine histidine, (3) aliphatic = glycine, alanine, valine, leucine, isoleucine, serine, threonine, with serine and threonine optionally be grouped separately as aliphatic-hydroxyl; (4) aromatic = phenylalanine, tyrosine, tryptophan; (5) amide = asparagine, glutamine; and (6) sulfur -containing = cysteine and methionine. (see, for example, *Biochemistry*, 2nd ed., Ed. by L. Stryer, WH Freeman and Co.: 1981). Whether a change in the amino acid sequence of a peptide results in a functional *hedgehog* homolog (e.g. functional in the sense that it acts to mimic or antagonize the wild-type form) can be readily determined by assessing the ability of the variant peptide to produce a response in cells in a fashion similar to the wild-type protein, or competitively inhibit such a response. Polypeptides in which more than one replacement has taken place can readily be tested in the same manner.

This invention further contemplates a method for generating sets of combinatorial mutants of the subject *hedgehog* proteins as well as truncation mutants, and is especially useful for identifying potential variant sequences (e.g. homologs) that are functional in binding to a receptor for *hedgehog* proteins. The purpose of screening such combinatorial libraries is to generate, for example, novel *hh* homologs which can act as either agonists or antagonist, or alternatively, possess novel activities all together. To illustrate, *hedgehog* homologs can be engineered by the present method to provide more efficient binding to a cognate receptor, yet still retain at least a portion of an activity associated with *hh*. Thus, combinatorially-derived homologs can be generated to have an increased potency relative to a naturally occurring form of the protein. Likewise, *hedgehog* homologs can be generated by the present combinatorial approach to act as antagonists, in that they are able to mimic, for example, binding to other extracellular matrix components (such as receptors), yet not induce any biological response, thereby inhibiting the action of authentic *hedgehog* or *hedgehog* agonists. Moreover, manipulation of certain domains of *hh* by the present method can provide domains more suitable for use in fusion proteins, such as one that incorporates portions of other proteins which are derived from the extracellular matrix and/or which bind extracellular matrix components.

In one aspect of this method, the amino acid sequences for a population of *hedgehog* homologs or other related proteins are aligned, preferably to promote the highest homology possible. Such a population of variants can include, for example, *hh* homologs from one or more species. Amino acids which appear at each position of the aligned sequences are selected to create a degenerate set of combinatorial sequences. In a preferred embodiment, the variegated library of *hedgehog* variants is generated by combinatorial mutagenesis at the nucleic acid level, and is encoded by a variegated gene library. For instance, a mixture of synthetic oligonucleotides can be enzymatically ligated into gene sequences such that the degenerate set of potential *hh* sequences are expressible as individual polypeptides, or alternatively, as a set of larger fusion proteins (e.g. for phage display) containing the set of *hh* sequences therein.

As illustrated in Figure 5A, to analyze the sequences of a population of variants, the amino acid sequences of interest can be aligned relative to sequence homology. The presence or absence of amino acids from an aligned sequence of a particular variant is relative to a chosen consensus length of a reference sequence, which can be real or artificial. In order to maintain the highest homology in alignment of sequences, deletions in the sequence of a variant relative to the reference sequence can be represented by an amino acid space (• or *), while insertional mutations in the variant relative to the reference sequence can be disregarded and left out of the sequence of the variant when aligned. For instance, Figure 5A includes the alignment of several cloned forms of *hh* from different species. Analysis of the alignment of the *hh* clones shown in Figure 5A can give rise to the generation of a degenerate library of polypeptides comprising potential *hh* sequences.

In an illustrative embodiment, alignment of exon 1/2 encoded sequences (e.g. the N-terminal approximately 165 residues of the mature protein) of each of the *Shh* clones produces a degenerate set of *Shh* polypeptides represented by the general formula:

C-G-P-G-R-G-X(1)-G-X(2)-R-R-H-P-K-K-L-T-P-L-A-Y-K-Q-F-I-P-N-V-A-E-K-T-L-G-A-S-G-R-Y-E-G-K-I-X(3)-R-N-S-E-R-F-K-E-L-T-P-N-Y-N-P-D-I-I-F-K-D-E-E-N-T-G-A-D-R-L-M-T-Q-R-C-K-D-K-L-N-X(4)-L-A-I-S-V-M-N-X(5)-W-P-G-V-X(6)-L-R-V-T-E-G-W-D-E-D-G-H-H-X(7)-E-E-S-L-H-Y-E-G-R-A-V-D-I-T-T-S-D-R-D-X(8)-S-K-Y-G-X(9)-L-X(10)-R-L-A-V-E-A-G-F-D-W-V-Y-Y-E-S-K-A-H-I-H-C-S-V-K-A-E (SEQ ID No: 40),

wherein each of the degenerate positions "X" can be an amino acid which occurs in that position in one of the human, mouse, chicken or zebrafish *Shh* clones, or, to expand the library, each X can also be selected from amongst amino acid residue which would be conservative substitutions for the amino acids which appear naturally in each of those positions. For instance, Xaa(1) represents Gly, Ala, Val, Leu, Ile, Phe, Tyr or Trp ; Xaa(2) represents Arg, His or Lys; Xaa(3) represents Gly, Ala, Val, Leu, Ile, Ser or Thr; Xaa(4)

38

represents Gly, Ala, Val, Leu, Ile, Ser or Thr; Xaa(5) represents Lys, Arg, His, Asn or Gln; Xaa(6) represents Lys, Arg or His; Xaa(7) represents Ser, Thr, Tyr, Trp or Phe; Xaa(8) represents Lys, Arg or His; Xaa(9) represents Met, Cys, Ser or Thr; and Xaa(10) represents Gly, Ala, Val, Leu, Ile, Ser or Thr. In an even more expansive library, each X can be selected from any amino acid.

In similar fashion, alignment of each of the human, mouse, chicken and zebrafish *hedgehog* clones (Figure 5B), can provide a degenerate polypeptide sequence represented by the general formula:

```

10      C-G-P-G-R-G-X(1)-X(2)-X(3)-R-R-X(4)-X(5)-X(6) -P-K-X(7)-L-X(8)-
      P-L-X(9)-Y-K-Q-F-X(10)-P-X(11)-X(12)-X(13)-E-X(14)-T-L-G-A-S-G-
      X(15)-X(16)-E-G-X(17)-X(18)-X(19) -R-X(20)-S-E-R-F-X(21)-X(22)-
      L-T-P-N-Y-N-P-D-I-I-F-K-D-E-E-N -X(23)-G-A-D-R-L-M-T-X(24)-R-C-
      K-X(25)-X(26)-X(27) -N-X(28)-L-A-I-S-V-M-N-X(29)-W-P-G-V-X(30)-
15      L-R-V-T-E-G-X(31) -D-E-D-G-H-H-X(32)-X(33)-X(34)-S-L-H-Y-E-G-R-
      A-X(35) -D-I-T-T-S-D-R-D-X(36)-X(37)-K-Y-G-X(38)-L-X(39)-R-L-A-
      V-E-A-G-F-D-W-V-Y-Y-E-S-X(40)-X(41)-H-X(42)-H-X(43)-S-V-K-X(44)
      -X(45) (SEQ ID No: 41),

```

wherein, as above, each of the degenerate positions "X" can be an amino acid which occurs in a corresponding position in one of the wild-type clones, and may also include amino acid residue which would be conservative substitutions, or each X can be any amino acid residue. In an exemplary embodiment, Xaa(1) represents Gly, Ala, Val, Leu, Ile, Pro, Phe or Tyr; Xaa(2) represents Gly, Ala, Val, Leu or Ile; Xaa(3) represents Gly, Ala, Val, Leu, Ile, Lys, His or Arg; Xaa(4) represents Lys, Arg or His; Xaa(5) represents Phe, Trp, Tyr or an amino acid gap; Xaa(6) represents Gly, Ala, Val, Leu, Ile or an amino acid gap; Xaa(7) represents Asn, Gln, His, Arg or Lys; Xaa(8) represents Gly, Ala, Val, Leu, Ile, Ser or Thr; Xaa(9) represents Gly, Ala, Val, Leu, Ile, Ser or Thr; Xaa(10) represents Gly, Ala, Val, Leu, Ile, Ser or Thr; Xaa(11) represents Ser, Thr, Gln or Asn; Xaa(12) represents Met, Cys, Gly, Ala, Val, Leu, Ile, Ser or Thr; Xaa(13) represents Gly, Ala, Val, Leu, Ile or Pro; Xaa(14) represents Arg, His or Lys; Xaa(15) represents Gly, Ala, Val, Leu, Ile, Pro, Arg, His or Lys; Xaa(16) represents Gly, Ala, Val, Leu, Ile, Phe or Tyr; Xaa(17) represents Arg, His or Lys; Xaa(18) represents Gly, Ala, Val, Leu, Ile, Ser or Thr; Xaa(19) represents Thr or Ser; Xaa(20) represents Gly, Ala, Val, Leu, Ile, Asn or Gln; Xaa(21) represents Arg, His or Lys; Xaa(22) represents Asp or Glu; Xaa(23) represents Ser or Thr; Xaa(24) represents Glu, Asp, Gln or Asn; Xaa(25) represents Glu or Asp; Xaa(26) represents Arg, His or Lys; Xaa(27) represents Gly, Ala, Val, Leu or Ile; Xaa(28) represents Gly, Ala, Val, Leu, Ile, Thr or Ser; Xaa(29) represents Met, Cys, Gln, Asn, Arg, Lys or His; Xaa(30) represents Arg, His or Lys; Xaa(31) represents Trp, Phe, Tyr, Arg, His or Lys; Xaa(32) represents Gly, Ala, Val, Leu, Ile, Ser, Thr, Tyr or Phe; Xaa(33) represents Gln, Asn, Asp or Glu; Xaa(34) represents Asp or Glu; Xaa(35) represents Gly, Ala, Val, Leu, or Ile; Xaa(36) represents Arg, His or Lys; Xaa(37) represents Asn, Gln, Thr or Ser; Xaa(38) represents Gly, Ala, Val, Leu, Ile, Ser, Thr, Met or

39

Cys; Xaa(39) represents Gly, Ala, Val, Leu, Ile, Thr or Ser; Xaa(40) represents Arg, His or Lys; Xaa(41) represents Asn, Gln, Gly, Ala, Val, Leu or Ile; Xaa(42) represents Gly, Ala, Val, Leu or Ile; Xaa(43) represents Gly, Ala, Val, Leu, Ile, Ser, Thr or Cys; Xaa(44) represents Gly, Ala, Val, Leu, Ile, Thr or Ser; and Xaa(45) represents Asp or Glu.

5 There are many ways by which the library of potential *hh* homologs can be generated from a degenerate oligonucleotide sequence. Chemical synthesis of a degenerate gene sequence can be carried out in an automatic DNA synthesizer, and the synthetic genes then ligated into an appropriate expression vector. The purpose of a degenerate set of genes is to provide, in one mixture, all of the sequences encoding the desired set of potential *hh* sequences. The synthesis of degenerate oligonucleotides is well known in the art (see for
10 example, Narang, SA (1983) *Tetrahedron* 39:3; Itakura et al. (1981) *Recombinant DNA, Proc 3rd Cleveland Sympos. Macromolecules*, ed. AG Walton, Amsterdam: Elsevier pp273-289; Itakura et al. (1984) *Annu. Rev. Biochem.* 53:323; Itakura et al. (1984) *Science* 198:1056; Ike et al. (1983) *Nucleic Acid Res.* 11:477. Such techniques have been employed
15 in the directed evolution of other proteins (see, for example, Scott et al. (1990) *Science* 249:386-390; Roberts et al. (1992) *PNAS* 89:2429-2433; Devlin et al. (1990) *Science* 249:404-406; Cwirla et al. (1990) *PNAS* 87: 6378-6382; as well as U.S. Patents Nos. 5,223,409, 5,198,346, and 5,096,815).

20 A wide range of techniques are known in the art for screening gene products of combinatorial libraries made by point mutations, and for screening cDNA libraries for gene products having a certain property. Such techniques will be generally adaptable for rapid screening of the gene libraries generated by the combinatorial mutagenesis of *hedgehog* homologs. The most widely used techniques for screening large gene libraries typically comprises cloning the gene library into replicable expression vectors, transforming
25 appropriate cells with the resulting library of vectors, and expressing the combinatorial genes under conditions in which detection of a desired activity facilitates relatively easy isolation of the vector encoding the gene whose product was detected. Each of the illustrative assays described below are amenable to high through-put analysis as necessary to screen large numbers of degenerate *hedgehog* sequences created by combinatorial mutagenesis
30 techniques.

35 In one embodiment, the combinatorial library is designed to be secreted (e.g. the polypeptides of the library all include a signal sequence but no transmembrane or cytoplasmic domains), and is used to transfect a eukaryotic cell that can be co-cultured with embryonic cells. A functional *hedgehog* protein secreted by the cells expressing the combinatorial library will diffuse to neighboring embryonic cells and induce a particular biological response, such as to illustrate, neuronal differentiation. Using antibodies directed to epitopes of particular neuronal cells (e.g. Islet-1 or Pax-1), the pattern of detection of

46

neuronal induction will resemble a gradient function, and will allow the isolation (generally after several repetitive rounds of selection) of cells producing active *hedgehog* homologs. Likewise, *hh* antagonists can be selected in similar fashion by the ability of the cell producing a functional antagonist to protect neighboring cells from the effect of wild-type *hedgehog* added to the culture media.

To illustrate, target cells are cultured in 24-well microtitre plates. Other eukaryotic cells are transfected with the combinatorial *hh* gene library and cultured in cell culture inserts (e.g. Collaborative Biomedical Products, Catalog #40446) that are able to fit into the wells of the microtitre plate. The cell culture inserts are placed in the wells such that recombinant *hh* homologs secreted by the cells in the insert can diffuse through the porous bottom of the insert and contact the target cells in the microtitre plate wells. After a period of time sufficient for functional forms of a *hedgehog* protein to produce a measurable response in the target cells, the inserts are removed and the effect of the variant *hedgehog* proteins on the target cells determined. For example, where the target cell is a neural crest cell and the activity desired from the *hh* homolog is the induction of neuronal differentiation, then fluorescently-labeled antibodies specific for Islet-1 or other neuronal markers can be used to score for induction in the target cells as indicative of a functional *hh* in that well. Cells from the inserts corresponding to wells which score positive for activity can be split and re-cultured on several inserts, the process being repeated until the active clones are identified.

In yet another screening assay, the candidate *hedgehog* gene products are displayed on the surface of a cell or viral particle, and the ability of particular cells or viral particles to associate with a *hedgehog*-binding moiety (such as an *hedgehog* receptor or a ligand which binds the *hedgehog* protein) via this gene product is detected in a "panning assay". Such panning steps can be carried out on cells cultured from embryos. For instance, the gene library can be cloned into the gene for a surface membrane protein of a bacterial cell, and the resulting fusion protein detected by panning (Ladner et al., WO 88/06630; Fuchs et al. (1991) *Bio/Technology* 9:1370-1371; and Goward et al. (1992) *TIBS* 18:136-140). In a similar fashion, fluorescently labeled molecules which bind *hh* can be used to score for potentially functional *hh* homologs. Cells can be visually inspected and separated under a fluorescence microscope, or, where the morphology of the cell permits, separated by a fluorescence-activated cell sorter.

In an alternate embodiment, the gene library is expressed as a fusion protein on the surface of a viral particle. For instance, in the filamentous phage system, foreign peptide sequences can be expressed on the surface of infectious phage, thereby conferring two significant benefits. First, since these phage can be applied to affinity matrices at very high concentrations, large number of phage can be screened at one time. Second, since each infectious phage displays the combinatorial gene product on its surface, if a particular phage

41

is recovered from an affinity matrix in low yield, the phage can be amplified by another round of infection. The group of almost identical *E. coli* filamentous phages M13, fd, and f1 are most often used in phage display libraries, as either of the phage gIII or gVIII coat proteins can be used to generate fusion proteins without disrupting the ultimate packaging of the viral particle (Ladner et al. PCT publication WO 90/02909; Garrard et al., PCT publication WO 92/09690; Marks et al. (1992) J. Biol. Chem. 267:16007-16010; Griffiths et al. (1993) EMBO J 12:725-734; Clackson et al. (1991) Nature 352:624-628; and Barbas et al. (1992) PNAS 89:4457-4461).

In an illustrative embodiment, the recombinant phage antibody system (RPAS, Pharmacia Catalog number 27-9400-01) can be easily modified for use in expressing and screening *hh* combinatorial libraries. For instance, the pCANTAB 5 phagemid of the RPAS kit contains the gene which encodes the phage gIII coat protein. The *hh* combinatorial gene library can be cloned into the phagemid adjacent to the gIII signal sequence such that it will be expressed as a gIII fusion protein. After ligation, the phagemid is used to transform competent *E. coli* TG1 cells. Transformed cells are subsequently infected with M13KO7 helper phage to rescue the phagemid and its candidate *hh* gene insert. The resulting recombinant phage contain phagemid DNA encoding a specific candidate *hh*, and display one or more copies of the corresponding fusion coat protein. The phage-displayed candidate *hedgehog* proteins which are capable of binding an *hh* receptor are selected or enriched by panning. For instance, the phage library can be applied to cultured embryonic cells and unbound phage washed away from the cells. The bound phage is then isolated, and if the recombinant phage express at least one copy of the wild type gIII coat protein, they will retain their ability to infect *E. coli*. Thus, successive rounds of reinfection of *E. coli*, and panning will greatly enrich for *hh* homologs, which can then be screened for further biological activities in order to differentiate agonists and antagonists. Moreover, differential panning, e.g., with two or more different *hh*-responsive cells, can facilitate isolation of *hedgehog* homologs of selectively narrower biological activity relative to the wild-type protein.

The invention also provides for reduction of the vertebrate *hh* protein to generate mimetics, e.g. peptide or non-peptide agents, which are able to disrupt binding of a vertebrate *hh* polypeptide of the present invention with an *hh* receptor. Thus, such mutagenic techniques as described above are also useful to map the determinants of the *hedgehog* proteins which participate in protein-protein interactions involved in, for example, binding of the subject vertebrate *hh* polypeptide to other extracellular matrix components. To illustrate, the critical residues of a subject *hh* polypeptide or *hh* ligand which are involved in molecular recognition of an *hh* receptor can be determined and used to generate *hedgehog*-derived peptidomimetics which competitively inhibit binding of the authentic *hedgehog* protein with that moiety. By employing, for example, scanning mutagenesis to map the amino acid

residues of each of the subject *hedgehog* proteins which are involved in binding other extracellular proteins, peptidomimetic compounds can be generated which mimic those residues of the *hedgehog* protein which facilitate the interaction. Such mimetics may then be used to interfere with the normal function of a *hedgehog* protein. For instance, non-
5 hydrolyzable peptide analogs of such residues can be generated using benzodiazepine (e.g., see Freidinger et al. in *Peptides: Chemistry and Biology*, G.R. Marshall ed., ESCOM Publisher: Leiden, Netherlands, 1988), azepine (e.g., see Huffman et al. in *Peptides: Chemistry and Biology*, G.R. Marshall ed., ESCOM Publisher: Leiden, Netherlands, 1988), substituted gamma lactam rings (Garvey et al. in *Peptides: Chemistry and Biology*, G.R.
10 Marshall ed., ESCOM Publisher: Leiden, Netherlands, 1988), keto-methylene pseudopeptides (Ewenson et al. (1986) *J Med Chem* 29:295; and Ewenson et al. in *Peptides: Structure and Function* (Proceedings of the 9th American Peptide Symposium) Pierce Chemical Co. Rockland, IL, 1985), β -turn dipeptide cores (Nagai et al. (1985) *Tetrahedron Lett* 26:647; and Sato et al. (1986) *J Chem Soc Perkin Trans* 1:1231), and β -aminoalcohols (Gordon et al.
15 (1985) *Biochem Biophys Res Commun* 126:419; and Dann et al. (1986) *Biochem Biophys Res Commun* 134:71).

Another aspect of the invention pertains to an antibody specifically reactive with a vertebrate *hedgehog* protein. For example, by using immunogens derived from *hedgehog* protein, e.g. based on the cDNA sequences, anti-protein/anti-peptide antisera or monoclonal
20 antibodies can be made by standard protocols (See, for example, *Antibodies: A Laboratory Manual* ed. by Harlow and Lane (Cold Spring Harbor Press: 1988)). A mammal, such as a mouse, a hamster or rabbit can be immunized with an immunogenic form of the peptide (e.g., a vertebrate *hh* polypeptide or an antigenic fragment which is capable of eliciting an antibody response). Techniques for conferring immunogenicity on a protein or peptide include
25 conjugation to carriers or other techniques well known in the art. An immunogenic portion of a *hedgehog* protein can be administered in the presence of adjuvant. The progress of immunization can be monitored by detection of antibody titers in plasma or serum. Standard ELISA or other immunoassays can be used with the immunogen as antigen to assess the levels of antibodies. In a preferred embodiment, the subject antibodies are immunospecific
30 for antigenic determinants of a *hedgehog* protein of a vertebrate organism, such as a mammal, e.g. antigenic determinants of a protein represented by SEQ ID Nos:8-14 or a closely related homolog (e.g. at least 85% homologous, preferably at least 90% homologous, and more preferably at least 95% homologous). In yet a further preferred embodiment of the present invention, in order to provide, for example, antibodies which are immuno-selective
35 for discrete *hedgehog* homologs, e.g. *Shh* versus *Dhh* versus *Ihh*, the anti-*hh* polypeptide antibodies do not substantially cross react (i.e. does not react specifically) with a protein which is, for example, less than 85% homologous to any of SEQ ID Nos:8-14; e.g., less than 95% homologous with one of SEQ ID Nos:8-14; e.g., less than 98-99% homologous with

one of SEQ ID Nos:8-14. By "not substantially cross react", it is meant that the antibody has a binding affinity for a non-homologous protein which is at least one order of magnitude, more preferably at least 2 orders of magnitude, and even more preferably at least 3 orders of magnitude less than the binding affinity of the antibody for one or more of the proteins of
5 SEQ ID Nos:8-14.

Following immunization of an animal with an antigenic preparation of a hedgehog protein, anti-*hh* antisera can be obtained and, if desired, polyclonal anti-*hh* antibodies isolated from the serum. To produce monoclonal antibodies, antibody-producing cells (lymphocytes) can be harvested from an immunized animal and fused by standard somatic cell fusion
10 procedures with immortalizing cells such as myeloma cells to yield hybridoma cells. Such techniques are well known in the art, and include, for example, the hybridoma technique (originally developed by Kohler and Milstein, (1975) *Nature*, 256: 495-497), the human B cell hybridoma technique (Kozbar et al., (1983) *Immunology Today*, 4: 72), and the EBV-hybridoma technique to produce human monoclonal antibodies (Cole et al., (1985)
15 *Monoclonal Antibodies and Cancer Therapy*, Alan R. Liss, Inc. pp. 77-96). Hybridoma cells can be screened immunochemically for production of antibodies specifically reactive with a vertebrate *hh* polypeptide of the present invention and monoclonal antibodies isolated from a culture comprising such hybridoma cells.

The term antibody as used herein is intended to include fragments thereof which are
20 also specifically reactive with one of the subject vertebrate *hh* polypeptides. Antibodies can be fragmented using conventional techniques and the fragments screened for utility in the same manner as described above for whole antibodies. For example, F(ab)₂ fragments can be generated by treating antibody with pepsin. The resulting F(ab)₂ fragment can be treated to reduce disulfide bridges to produce Fab fragments. The antibody of the present invention
25 is further intended to include bispecific and chimeric molecules having affinity for a hedgehog protein conferred by at least one CDR region of the antibody.

Both monoclonal and polyclonal antibodies (Ab) directed against authentic *hedgehog* polypeptides, or *hedgehog* variants, and antibody fragments such as Fab and F(ab)₂, can be used to block the action of one or more *hedgehog* proteins and allow the study of the role of
30 these proteins in, for example, embryogenesis and/or maintenance of differential tissue. For example, purified monoclonal Abs can be injected directly into the limb buds of chick or mouse embryos. It is demonstrated in the examples below that *hh* is expressed in the limb buds of, for example, day 10.5 embryos. Thus, the use of anti-*hh* Abs during this developmental stage can allow assessment of the effect of *hh* on the formation of limbs *in vivo*. In a similar approach, hybridomas producing anti-*hh* monoclonal Abs, or biodegradable gels in which anti-*hh* Abs are suspended, can be implanted at a site proximal
35 or within the area at which *hh* action is intended to be blocked. Experiments of this nature

can aid in deciphering the role of this and other factors that may be involved in limb patterning and tissue formation.

Antibodies which specifically bind *hedgehog* epitopes can also be used in immunohistochemical staining of tissue samples in order to evaluate the abundance and pattern of expression of each of the subject *hh* polypeptides. Anti-*hedgehog* antibodies can be used diagnostically in immuno-precipitation and immuno-blotting to detect and evaluate *hedgehog* protein levels in tissue as part of a clinical testing procedure. For instance, such measurements can be useful in predictive valuations of the onset or progression of neurological disorders, such as those marked by denervation-like or disuse-like symptoms. Likewise, the ability to monitor *hh* levels in an individual can allow determination of the efficacy of a given treatment regimen for an individual afflicted with such a disorder. The level of *hh* polypeptides may be measured in bodily fluid, such as in samples of cerebral spinal fluid or amniotic fluid, or can be measured in tissue, such as produced by biopsy. Diagnostic assays using anti-*hh* antibodies can include, for example, immunoassays designed to aid in early diagnosis of a neurodegenerative disorder, particularly ones which are manifest at birth. Diagnostic assays using anti-*hh* polypeptide antibodies can also include immunoassays designed to aid in early diagnosis and phenotyping of a differentiative disorder, as well as neoplastic or hyperplastic disorders.

Another application of anti-*hh* antibodies of the present invention is in the immunological screening of cDNA libraries constructed in expression vectors such as λ gt11, λ gt18-23, λ ZAP, and λ ORF8. Messenger libraries of this type, having coding sequences inserted in the correct reading frame and orientation, can produce fusion proteins. For instance, λ gt11 will produce fusion proteins whose amino termini consist of β -galactosidase amino acid sequences and whose carboxy termini consist of a foreign polypeptide. Antigenic epitopes of an *hh* protein, e.g. other orthologs of a particular *hedgehog* protein or other homologs from the same species, can then be detected with antibodies, as, for example, reacting nitrocellulose filters lifted from infected plates with anti-*hh* antibodies. Positive phage detected by this assay can then be isolated from the infected plate. Thus, the presence of *hedgehog* homologs can be detected and cloned from other animals, as can alternate isoforms (including splicing variants) from humans.

Moreover, the nucleotide sequences determined from the cloning of *hh* genes from vertebrate organisms will further allow for the generation of probes and primers designed for use in identifying and/or cloning *hedgehog* homologs in other cell types, e.g. from other tissues, as well as *hh* homologs from other vertebrate organisms. For instance, the present invention also provides a probe/primer comprising a substantially purified oligonucleotide, which oligonucleotide comprises a region of nucleotide sequence that hybridizes under stringent conditions to at least 10 consecutive nucleotides of sense or anti-sense sequence

45

selected from the group consisting of SEQ ID No:1, SEQ ID No:2, SEQ ID No:3, SEQ ID No:4, SEQ ID No:5, SEQ ID No:6 and SEQ ID No:7, or naturally occurring mutants thereof. For instance, primers based on the nucleic acid represented in SEQ ID Nos:1-7 can be used in PCR reactions to clone *hedgehog* homologs. Likewise, probes based on the subject *hedgehog* sequences can be used to detect transcripts or genomic sequences encoding the same or homologous proteins. In preferred embodiments, the probe further comprises a label group attached thereto and able to be detected, e.g. the label group is selected from the group consisting of radioisotopes, fluorescent compounds, enzymes, and enzyme co-factors.

Such probes can also be used as a part of a diagnostic test kit for identifying cells or tissue which misexpress a *hedgehog* protein, such as by measuring a level of a *hedgehog* encoding nucleic acid in a sample of cells from a patient; e.g. detecting *hh* mRNA levels or determining whether a genomic *hh* gene has been mutated or deleted.

To illustrate, nucleotide probes can be generated from the subject *hedgehog* genes which facilitate histological screening of intact tissue and tissue samples for the presence (or absence) of *hedgehog*-encoding transcripts. Similar to the diagnostic uses of anti-*hedgehog* antibodies, the use of probes directed to *hh* messages, or to genomic *hh* sequences, can be used for both predictive and therapeutic evaluation of allelic mutations which might be manifest in, for example, neoplastic or hyperplastic disorders (e.g. unwanted cell growth) or abnormal differentiation of tissue. Used in conjunction with immunoassays as described above, the oligonucleotide probes can help facilitate the determination of the molecular basis for a developmental disorder which may involve some abnormality associated with expression (or lack thereof) of a *hedgehog* protein. For instance, variation in polypeptide synthesis can be differentiated from a mutation in a coding sequence.

Accordingly, the present method provides a method for determining if a subject is at risk for a disorder characterized by aberrant control of differentiation or unwanted cell proliferation. For instance, the subject assay can be used in the screening and diagnosis of genetic and acquired disorders which involve alteration in one or more of the *hedgehog* genes. In preferred embodiments, the subject method can be generally characterized as comprising: detecting, in a tissue sample of the subject (e.g. a human patient), the presence or absence of a genetic lesion characterized by at least one of (i) a mutation of a gene encoding a *hedgehog* protein or (ii) the mis-expression of a *hedgehog* gene. To illustrate, such genetic lesions can be detected by ascertaining the existence of at least one of (i) a deletion of one or more nucleotides from a *hedgehog* gene, (ii) an addition of one or more nucleotides to a *hedgehog* gene, (iii) a substitution of one or more nucleotides of a *hedgehog* gene, (iv) a gross chromosomal rearrangement of a *hedgehog* gene, (v) a gross alteration in the level of a messenger RNA transcript of an *hh* gene, (vi) the presence of a non-wild type splicing pattern of a messenger RNA transcript of a vertebrate *hh* gene, and (vii) a non-wild

type level of a *hedgehog* protein. In one aspect of the invention there is provided a probe/primer comprising an oligonucleotide containing a region of nucleotide sequence which is capable of hybridizing to a sense or antisense sequence selected from the group consisting of SEQ ID Nos:1-7, or naturally occurring mutants thereof, or 5' or 3' flanking sequences or intronic sequences naturally associated with a vertebrate *hh* gene. The probe is exposed to nucleic acid of a tissue sample; and the hybridization of the probe to the sample nucleic acid is detected. In certain embodiments, detection of the lesion comprises utilizing the probe/primer in a polymerase chain reaction (PCR) (see, e.g., U.S. Patent No: 4,683,195 and 4,683,202) or, alternatively, in a ligation chain reaction (LCR) (see, e.g., Landegran et al. (1988) *Science*, 241:1077-1080; and NaKazawa et al. (1944) *PNAS* 91:360-364) the later of which can be particularly useful for detecting point mutations in *hedgehog* genes. Alternatively, immunoassays can be employed to determine the level of *hh* proteins, either soluble or membrane bound.

Yet another diagnostic screen employs a source of *hedgehog* protein directly. As described herein, *hedgehog* proteins of the present invention are involved in the induction of differentiation. Accordingly, the pathology of certain differentiative and/or proliferative disorders can be marked by loss of *hedgehog* sensitivity by the afflicted tissue. Consequently, the response of a tissue or cell sample to an inductive amount of a *hedgehog* protein can be used to detect and characterize certain cellular transformations and degenerative conditions. For instance, tissue/cell samples from a patient can be treated with a *hedgehog* agonist and the response of the tissue to the treatment determined. Response can be qualified and/or quantified, for example, on the basis of phenotypic change as result of *hedgehog* induction. For example, expression of gene products induced by *hedgehog* treatment can be scored for by immunoassay. The *patched* protein, for example, is upregulated in drosophila in response to Dros-HH, and, in light of the findings herein, a presumed vertebrate homolog will similarly be upregulated. Thus, detection of *patched* expression on the cells of the patient sample can permit detection of tissue that is not *hedgehog*-responsive. Likewise, scoring for other phenotypic markers provides a means for determining the response to *hedgehog*.

Furthermore, by making available purified and recombinant *hedgehog* polypeptides, the present invention facilitates the development of assays which can be used to screen for drugs, including *hedgehog* homologs, which are either agonists or antagonists of the normal cellular function of the subject *hedgehog* polypeptides, or of their role in the pathogenesis of cellular differentiation and/or proliferation and disorders related thereto. In one embodiment, the assay evaluates the ability of a compound to modulate binding between a *hedgehog* polypeptide and a *hedgehog* receptor. A variety of assay formats will suffice and, in light of the present inventions, will be comprehended by skilled artisan.

In many drug screening programs which test libraries of compounds and natural extracts, high throughput assays are desirable in order to maximize the number of compounds surveyed in a given period of time. Assays which are performed in cell-free systems, such as may be derived with purified or semi-purified proteins, are often preferred as "primary" screens in that they can be generated to permit rapid development and relatively easy detection of an alteration in a molecular target which is mediated by a test compound. Moreover, the effects of cellular toxicity and/or bioavailability of the test compound can be generally ignored in the *in vitro* system, the assay instead being focused primarily on the effect of the drug on the molecular target as may be manifest in an alteration of binding affinity with receptor proteins. Accordingly, in an exemplary screening assay of the present invention, the compound of interest is contacted with a *hedgehog* receptor polypeptide which is ordinarily capable of binding a *hedgehog* protein. To the mixture of the compound and receptor is then added a composition containing a *hedgehog* polypeptide. Detection and quantification of receptor/*hedgehog* complexes provides a means for determining the compound's efficacy at inhibiting (or potentiating) complex formation between the receptor protein and the *hedgehog* polypeptide. The efficacy of the compound can be assessed by generating dose response curves from data obtained using various concentrations of the test compound. Moreover, a control assay can also be performed to provide a baseline for comparison. In the control assay, isolated and purified *hedgehog* polypeptide is added to a composition containing the receptor protein, and the formation of receptor/*hedgehog* complex is quantitated in the absence of the test compound.

In an illustrative embodiment, the polypeptide utilized as a *hedgehog* receptor can be generated from the drosophila *patched* protein or a vertebrate homolog thereof. In light of the ability of, for example, *Shh* to activate Dros-HH pathways in transgenic drosophila (see Example 4), it may be concluded that vertebrate *hedgehog* proteins are capable of binding to Drosophila HH receptors. Accordingly, an exemplary screening assay includes a suitable portion of the *patched* protein (SEQ ID No. 42), such as one or both of the substantial extracellular domains (e.g. residues Lys-93 to His-426 and Arg-700 to Arg-966). For instance, the *patched* protein can be provided in soluble form, as for example a preparation of one of the extracellular domains, or a preparation of both of the extracellular domains which are covalently connected by an unstructured linker (see, for example, Huston et al. (1988) PNAS 85:4879; and U.S. Patent No. 5,091,513), or can be provided as part of a liposomal preparation or expressed on the surface of a cell.

Complex formation between the *hedgehog* polypeptide and a *hedgehog* receptor may be detected by a variety of techniques. For instance, modulation of the formation of complexes can be quantitated using, for example, detectably labeled proteins such as

radiolabelled, fluorescently labeled, or enzymatically labeled *hedgehog* polypeptides, by immunoassay, or by chromatographic detection.

Typically, it will be desirable to immobilize either the *hedgehog* receptor or the *hedgehog* polypeptide to facilitate separation of receptor/*hedgehog* complexes from uncomplexed forms of one of the proteins, as well as to accommodate automation of the assay. In one embodiment, a fusion protein can be provided which adds a domain that allows the protein to be bound to a matrix. For example, glutathione-S-transferase/receptor (GST/receptor) fusion proteins can be adsorbed onto glutathione sepharose beads (Sigma Chemical, St. Louis, MO) or glutathione derivatized microtitre plates, which are then combined with the *hedgehog* polypeptide, e.g. an ³⁵S-labeled *hedgehog* polypeptide, and the test compound and incubated under conditions conducive to complex formation, e.g. at physiological conditions for salt and pH, though slightly more stringent conditions may be desired. Following incubation, the beads are washed to remove any unbound *hedgehog* polypeptide, and the matrix bead-bound radiolabel determined directly (e.g. beads placed in scintillant), or in the supernatant after the receptor/*hedgehog* complexes are dissociated. Alternatively, the complexes can dissociated from the bead, separated by SDS-PAGE gel, and the level of *hedgehog* polypeptide found in the bead fraction quantitated from the gel using standard electrophoretic techniques.

Other techniques for immobilizing proteins on matrices are also available for use in the subject assay. For instance, soluble portions of the *hedgehog* receptor protein can be immobilized utilizing conjugation of biotin and streptavidin. For instance, biotinylated receptor molecules can be prepared from biotin-NHS (N-hydroxy-succinimide) using techniques well known in the art (e.g., biotinylation kit, Pierce Chemicals, Rockford, IL), and immobilized in the wells of streptavidin-coated 96 well plates (Pierce Chemical). Alternatively, antibodies reactive with the *hedgehog* receptor but which do not interfere with *hedgehog* binding can be derivatized to the wells of the plate, and the receptor trapped in the wells by antibody conjugation. As above, preparations of a *hedgehog* polypeptide and a test compound are incubated in the receptor-presenting wells of the plate, and the amount of receptor/*hedgehog* complex trapped in the well can be quantitated. Exemplary methods for detecting such complexes, in addition to those described above for the GST-immobilized complexes, include immunodetection of complexes using antibodies reactive with the *hedgehog* polypeptide, or which are reactive with the receptor protein and compete for binding with the *hedgehog* polypeptide; as well as enzyme-linked assays which rely on detecting an enzymatic activity associated with the *hedgehog* polypeptide. In the instance of the latter, the enzyme can be chemically conjugated or provided as a fusion protein with the *hedgehog* polypeptide. To illustrate, the *hedgehog* polypeptide can be chemically cross-linked or genetically fused with alkaline phosphatase, and the amount of *hedgehog*

polypeptide trapped in the complex can be assessed with a chromogenic substrate of the enzyme, e.g. paranitrophenylphosphate. Likewise, a fusion protein comprising the *hedgehog* polypeptide and glutathione-S-transferase can be provided, and complex formation quantitated by detecting the GST activity using 1-chloro-2,4-dinitrobenzene (Habig et al (1974) *J Biol Chem* 249:7130).

For processes which rely on immunodetection for quantitating one of the proteins trapped in the complex, antibodies against the protein, such as the anti-*hedgehog* antibodies described herein, can be used. Alternatively, the protein to be detected in the complex can be "epitope tagged" in the form of a fusion protein which includes, in addition to the *hedgehog* polypeptide or *hedgehog* receptor sequence, a second polypeptide for which antibodies are readily available (e.g. from commercial sources). For instance, the GST fusion proteins described above can also be used for quantification of binding using antibodies against the GST moiety. Other useful epitope tags include myc-epitopes (e.g., see Ellison et al. (1991) *J Biol Chem* 266:21150-21157) which includes a 10-residue sequence from c-myc, as well as the pFLAG system (International Biotechnologies, Inc.) or the pEZZ-protein A system (Pharmacia, NJ).

Where the desired portion of the *hh* receptor (or other *hedgehog* binding molecule) cannot be provided in soluble form, liposomal vesicles can be used to provide manipulatable and isolatable sources of the receptor. For example, both authentic and recombinant forms of the *patched* protein can be reconstituted in artificial lipid vesicles (e.g. phosphatidylcholine liposomes) or in cell membrane-derived vesicles (see, for example, Bear et al. (1992) *Cell* 68:809-818; Newton et al. (1983) *Biochemistry* 22:6110-6117; and Reber et al. (1987) *J Biol Chem* 262:11369-11374).

In addition to cell-free assays, such as described above, the readily available source of vertebrate *hedgehog* proteins provided by the present invention also facilitates the generation of cell-based assays for identifying small molecule agonists/antagonists and the like. Analogous to the cell-based assays described above for screening combinatorial libraries, cells which are sensitive to *hedgehog* induction can be contacted with a *hedgehog* protein and a test agent of interest, with the assay scoring for modulation in *hedgehog* inductive responses by the target cell in the presence and absence of the test agent. As with the cell-free assays, agents which produce a statistically significant change in *hedgehog* activities (either inhibition or potentiation) can be identified. In an illustrative embodiment, motor neuron progenitor cells, such as from neural plate explants, can be used as target cells. Treatment of such explanted cells with, for example, *Shh* causes the cells to differentiate into motor neurons. By detecting the co-expression of the LIM homeodomain protein Islet-1 (Thor et al. (1991) *Neuron* 7:881-889; Ericson et al. (1992) *Science* 256:1555-1560) and the immunoglobulin-like protein SC1 (Tanaka et al. (1984) *Dev Biol* 106:26-37), the ability of a

candidate agent to potentiate or inhibit *Shh* induction of motor neuron differentiation can be measured. The *hedgehog* protein can be provided as a purified source, or in the form of cells/tissue which express the protein and which are co-cultured with the target cells.

In yet another embodiment, the method of the present invention can be used to isolate and clone *hedgehog* receptors. For example, purified *hedgehog* proteins of the present invention can be employed to precipitate *hedgehog* receptor proteins from cell fractions prepared from cells which are responsive to a *hedgehog* protein. For instance, purified *hedgehog* protein can be derivatized with biotin (using, for instance, NHS-Biotin, Pierce Chemical catalog no. 21420G), and the biotinylated protein utilized to saturate membrane bound *hh* receptors. The *hedgehog* bound receptors can subsequently be adsorbed or immobilized on streptavidin. If desired, the *hedgehog*-receptor complex can be cross-linked with a chemical cross-linking agent. In such a manner, *hh* receptors can be purified, preferably to near homogeneity. The isolated *hh* receptor can then be partially digested with, for example, trypsin, and the resulting peptides separated by reverse-phase chromatography. The chromatography fragments are then analyzed by Edman degradation to obtain single sequences for two or more of the proteolytic fragments. From the chemically determined amino acid sequence for each of these tryptic fragments, a set of oligonucleotide primers can be designed for PCR. These primers can be used to screen both genomic and cDNA libraries. Similar strategies for cloning receptors have been employed, for example, to obtain the recombinant gene for somatostatin receptors (Eppler et al. (1992) *J Biol Chem* 267:15603-15612).

Other techniques for identifying *hedgehog* receptors by expression cloning will be evident in light of the present disclosure. For instance, purified *hh* polypeptides can be immobilized in wells of micro titre plates and contacted with, for example, COS cells transfected with a cDNA library (e.g., from tissue expected to be responsive to *hedgehog* induction). From this panning assay, cells which express *hedgehog* receptor molecules can be isolated on the basis of binding to the immobilized *hedgehog* protein. Another cloning system, described in PCT publications WO 92/06220 of Flanagan and Leder, involves the use of an expression cloning system whereby a *hedgehog* receptor is stored on the basis of binding to a *hedgehog*/alkaline phosphatase fusion protein (see also Cheng et al. (1994) *Cell* 79:157-168).

Another aspect of the present invention relates to a method of inducing and/or maintaining a differentiated state, enhancing survival, and/or promoting proliferation of a cell responsive to a vertebrate *hedgehog* protein, by contacting the cells with an *hh* agonist or an *hh* antagonist as the circumstances may warrant. For instance, it is contemplated by the invention that, in light of the present finding of an apparently broad involvement of *hedgehog* proteins in the formation of ordered spatial arrangements of differentiated tissues in

51

vertebrates, the subject method could be used to generate and/or maintain an array of different vertebrate tissue both *in vitro* and *in vivo*. The *hh* agent, whether inductive or anti-inductive, can be, as appropriate, any of the preparations described above, including isolated polypeptides, gene therapy constructs, antisense molecules, peptidomimetics or agents identified in the drug assays provided herein. Moreover, it is contemplated that, based on the observation of activity of the vertebrate *hedgehog* proteins in *Drosophila*, *hh* agents, for purposes of therapeutic and diagnostic uses, can include the Dros-HH protein and homologs thereof. Moreover, the source of *hedgehog* protein can be, in addition to purified protein or recombinant cells, cells or tissue explants which naturally produce one or more *hedgehog* proteins. For instance, as described in Example 2, neural tube explants from embryos, particularly floorplate tissue, can provide a source for *Shh* polypeptide, which source can be implanted in a patient or otherwise provided, as appropriate, for induction or maintenance of differentiation.

For example, the present method is applicable to cell culture techniques. *In vitro* neuronal culture systems have proved to be fundamental and indispensable tools for the study of neural development, as well as the identification of neurotrophic factors such as nerve growth factor (NGF), ciliary trophic factors (CNTF), and brain derived neurotrophic factor (BDNF). Once a neuronal cell has become terminally-differentiated it typically will not change to another terminally differentiated cell-type. However, neuronal cells can nevertheless readily lose their differentiated state. This is commonly observed when they are grown in culture from adult tissue, and when they form a blastema during regeneration. The present method provides a means for ensuring an adequately restrictive environment in order to maintain neuronal cells at various stages of differentiation, and can be employed, for instance, in cell cultures designed to test the specific activities of other trophic factors. In such embodiments of the subject method, the cultured cells can be contacted with an *hh* polypeptide, or an agent identified in the assays described above, in order to induce neuronal differentiation (e.g. of a stem cell), or to maintain the integrity of a culture of terminally-differentiated neuronal cells by preventing loss of differentiation. The source of *hedgehog* protein in the culture can be derived from, for example, a purified or semi-purified protein composition added directly to the cell culture media, or alternatively, supported and/or released from a polymeric device which supports the growth of various neuronal cells and which has been doped with the protein. The source of the *hedgehog* protein can also be a cell that is co-cultured with the intended neuronal cell and which produces a recombinant or wild-type *hedgehog* protein. Alternatively, the source can be the neuronal cell itself which has been engineered to produce a recombinant *hedgehog* protein. In an exemplary embodiment, a naive neuronal cell (e.g. a stem cell) is treated with an *hh* agonist in order to induce differentiation of the cells into, for example, sensory neurons or, alternatively, motoneurons. Such neuronal cultures can be used as convenient assay systems as well as

sources of implantable cells for therapeutic treatments. For example, *hh* polypeptides may be useful in establishing and maintaining the olfactory neuron cultures described in U.S. Patent 5,318,907 and the like.

According to the present invention, large numbers of non-tumorigenic neural progenitor cells can be perpetuated *in vitro* and induced to differentiate by contact with *hedgehog* proteins. Generally, a method is provided comprising the steps of isolating neural progenitor cells from an animal, perpetuating these cells *in vitro* or *in vivo*, preferably in the presence of growth factors, and differentiating these cells into particular neural phenotypes, e.g., neurons and glia, by contacting the cells with a *hedgehog* agonist.

Progenitor cells are thought to be under a tonic inhibitory influence which maintains the progenitors in a suppressed state until their differentiation is required. However, recent techniques have been provided which permit these cells to be proliferated, and unlike neurons which are terminally differentiated and therefore non-dividing, they can be produced in unlimited number and are highly suitable for transplantation into heterologous and autologous hosts with neurodegenerative diseases.

By "progenitor" it is meant an oligopotent or multipotent stem cell which is able to divide without limit and, under specific conditions, can produce daughter cells which terminally differentiate such as into neurons and glia. These cells can be used for transplantation into a heterologous or autologous host. By heterologous is meant a host other than the animal from which the progenitor cells were originally derived. By autologous is meant the identical host from which the cells were originally derived.

Cells can be obtained from embryonic, post-natal, juvenile or adult neural tissue from any animal. By any animal is meant any multicellular animal which contains nervous tissue. More particularly, is meant any fish, reptile, bird, amphibian or mammal and the like. The most preferable donors are mammals, especially mice and humans.

In the case of a heterologous donor animal, the animal may be euthanized, and the brain and specific area of interest removed using a sterile procedure. Brain areas of particular interest include any area from which progenitor cells can be obtained which will serve to restore function to a degenerated area of the host's brain. These regions include areas of the central nervous system (CNS) including the cerebral cortex, cerebellum, midbrain, brainstem, spinal cord and ventricular tissue, and areas of the peripheral nervous system (PNS) including the carotid body and the adrenal medulla. More particularly, these areas include regions in the basal ganglia, preferably the striatum which consists of the caudate and putamen, or various cell groups such as the globus pallidus, the subthalamic nucleus, the nucleus basalis which is found to be degenerated in Alzheimer's Disease patients, or the substantia nigra pars compacta which is found to be degenerated in Parkinson's Disease patients.

53

Human heterologous neural progenitor cells may be derived from fetal tissue obtained from elective abortion, or from a post-natal, juvenile or adult organ donor. Autologous neural tissue can be obtained by biopsy, or from patients undergoing neurosurgery in which neural tissue is removed, in particular during epilepsy surgery, and more particularly during temporal lobectomies and hippocampalectomies.

Cells can be obtained from donor tissue by dissociation of individual cells from the connecting extracellular matrix of the tissue. Dissociation can be obtained using any known procedure, including treatment with enzymes such as trypsin, collagenase and the like, or by using physical methods of dissociation such as with a blunt instrument. Dissociation of fetal cells can be carried out in tissue culture medium, while a preferable medium for dissociation of juvenile and adult cells is artificial cerebral spinal fluid (aCSF). Regular aCSF contains 124 mM NaCl, 5 mM KCl, 1.3 mM MgCl₂, 2 mM CaCl₂, 26 mM NaHCO₃, and 10 mM D-glucose. Low Ca²⁺ aCSF contains the same ingredients except for MgCl₂ at a concentration of 3.2 mM and CaCl₂ at a concentration of 0.1 mM.

Dissociated cells can be placed into any known culture medium capable of supporting cell growth, including MEM, DMEM, RPMI, F-12, and the like, containing supplements which are required for cellular metabolism such as glutamine and other amino acids, vitamins, minerals and useful proteins such as transferrin and the like. Medium may also contain antibiotics to prevent contamination with yeast, bacteria and fungi such as penicillin, streptomycin, gentamicin and the like. In some cases, the medium may contain serum derived from bovine, equine, chicken and the like. A particularly preferable medium for cells is a mixture of DMEM and F-12.

Conditions for culturing should be close to physiological conditions. The pH of the culture media should be close to physiological pH, preferably between pH 6-8, more preferably close to pH 7, even more particularly about pH 7.4. Cells should be cultured at a temperature close to physiological temperature, preferably between 30°C-40°C, more preferably between 32°C-38°C, and most preferably between 35°C-37°C.

Cells can be grown in suspension or on a fixed substrate, but proliferation of the progenitors is preferably done in suspension to generate large numbers of cells by formation of "neurospheres" (see, for example, Reynolds et al. (1992) *Science* 255:1070-1709; and PCT Publications WO93/01275, WO94/09119, WO94/10292, and WO94/16718). In the case of propagating (or splitting) suspension cells, flasks are shaken well and the neurospheres allowed to settle on the bottom corner of the flask. The spheres are then transferred to a 50 ml centrifuge tube and centrifuged at low speed. The medium is aspirated, the cells resuspended in a small amount of medium with growth factor, and the cells mechanically dissociated and resuspended in separate aliquots of media.

54

Cell suspensions in culture medium are supplemented with any growth factor which allows for the proliferation of progenitor cells and seeded in any receptacle capable of sustaining cells, though as set out above, preferably in culture flasks or roller bottles. Cells typically proliferate within 3-4 days in a 37°C incubator, and proliferation can be reinitiated
5 at any time after that by dissociation of the cells and resuspension in fresh medium containing growth factors.

In the absence of substrate, cells lift off the floor of the flask and continue to proliferate in suspension forming a hollow sphere of undifferentiated cells. After approximately 3-10 days *in vitro*, the proliferating clusters (neurospheres) are fed every 2-7
10 days, and more particularly every 2-4 days by gentle centrifugation and resuspension in medium containing growth factor.

After 6-7 days *in vitro*, individual cells in the neurospheres can be separated by physical dissociation of the neurospheres with a blunt instrument, more particularly by triturating the neurospheres with a pipette. Single cells from the dissociated neurospheres are
15 suspended in culture medium containing growth factors, and differentiation of the cells can be induced by plating (or resuspending) the cells in the presence of a *hedgehog* agonist, and (optionally) any other factor capable of sustaining differentiation, such as bFGF and the like.

To further illustrate other uses of *hedgehog* agonists and antagonists, it is noted that intracerebral grafting has emerged as an additional approach to central nervous system
20 therapies. For example, one approach to repairing damaged brain tissues involves the transplantation of cells from fetal or neonatal animals into the adult brain (Dunnett et al. (1987) *J Exp Biol* 123:265-289; and Freund et al. (1985) *J Neurosci* 5:603-616). Fetal neurons from a variety of brain regions can be successfully incorporated into the adult brain, and such grafts can alleviate behavioral defects. For example, movement disorder induced by
25 lesions of dopaminergic projections to the basal ganglia can be prevented by grafts of embryonic dopaminergic neurons. Complex cognitive functions that are impaired after lesions of the neocortex can also be partially restored by grafts of embryonic cortical cells. The use of *hedgehog* proteins or mimetics, such as *Shh* or *Dhh*, in the culture can prevent loss of differentiation, or where fetal tissue is used, especially neuronal stem cells, can be
30 used to induce differentiation.

Stem cells useful in the present invention are generally known. For example, several neural crest cells have been identified, some of which are multipotent and likely represent uncommitted neural crest cells, and others of which can generate only one type of cell, such as sensory neurons, and likely represent committed progenitor cells. The role of *hedgehog*
35 proteins employed in the present method to culture such stem cells can be to induce differentiation of the uncommitted progenitor and thereby give rise to a committed progenitor

55

cell, or to cause further restriction of the developmental fate of a committed progenitor cell towards becoming a terminally-differentiated neuronal cell. For example, the present method can be used *in vitro* to induce and/or maintain the differentiation of neural crest cells into glial cells, schwann cells, chromaffin cells, cholinergic sympathetic or parasympathetic neurons, as well as peptidergic and serotonergic neurons. The *hedgehog* protein can be used alone, or can be used in combination with other neurotrophic factors which act to more particularly enhance a particular differentiation fate of the neuronal progenitor cell. In the later instance, an *hh* polypeptide might be viewed as ensuring that the treated cell has achieved a particular phenotypic state such that the cell is poised along a certain developmental pathway so as to be properly induced upon contact with a secondary neurotrophic factor. In similar fashion, even relatively undifferentiated stem cells or primitive neuroblasts can be maintained in culture and caused to differentiate by treatment with *hedgehog* agonists. Exemplary primitive cell cultures comprise cells harvested from the neural plate or neural tube of an embryo even before much overt differentiation has occurred.

In addition to the implantation of cells cultured in the presence of a functional *hedgehog* activity and other *in vitro* uses described above, yet another aspect of the present invention concerns the therapeutic application of a *hedgehog* protein or mimetic to enhance survival of neurons and other neuronal cells in both the central nervous system and the peripheral nervous system. The ability of *hedgehog* protein to regulate neuronal differentiation during development of the nervous system and also presumably in the adult state indicates that certain of the *hedgehog* proteins can be reasonably expected to facilitate control of adult neurons with regard to maintenance, functional performance, and aging of normal cells; repair and regeneration processes in chemically or mechanically lesioned cells; and prevention of degeneration and premature death which result from loss of differentiation in certain pathological conditions. In light of this understanding, the present invention specifically contemplates applications of the subject method to the treatment of (prevention and/or reduction of the severity of) neurological conditions deriving from: (i) acute, subacute, or chronic injury to the nervous system, including traumatic injury, chemical injury, vasal injury and deficits (such as the ischemia resulting from stroke), together with infectious/inflammatory and tumor-induced injury; (ii) aging of the nervous system including Alzheimer's disease; (iii) chronic neurodegenerative diseases of the nervous system, including Parkinson's disease, Huntington's chorea, amyotrophic lateral sclerosis and the like, as well as spinocerebellar degenerations; and (iv) chronic immunological diseases of the nervous system or affecting the nervous system, including multiple sclerosis.

Many neurological disorders are associated with degeneration of discrete populations of neuronal elements and may be treatable with a therapeutic regimen which includes a *hedgehog* agonist. For example, Alzheimer's disease is associated with deficits in several

neurotransmitter systems, both those that project to the neocortex and those that reside with the cortex. For instance, the nucleus basalis in patients with Alzheimer's disease have been observed to have a profound (75%) loss of neurons compared to age-matched controls. Although Alzheimer's disease is by far the most common form of dementia, several other disorders can produce dementia. Several of these are degenerative diseases characterized by the death of neurons in various parts of the central nervous system, especially the cerebral cortex. However, some forms of dementia are associated with degeneration of the thalamus or the white matter underlying the cerebral cortex. Here, the cognitive dysfunction results from the isolation of cortical areas by the degeneration of efferents and afferents. Huntington's disease involves the degeneration of intrastriatal and cortical cholinergic neurons and GABAergic neurons. Pick's disease is a severe neuronal degeneration in the neocortex of the frontal and anterior temporal lobes, sometimes accompanied by death of neurons in the striatum. Treatment of patients suffering from such degenerative conditions can include the application of *hedgehog* polypeptides, or agents which mimic their effects, in order to control, for example, differentiation and apoptotic events which give rise to loss of neurons (e.g. to enhance survival of existing neurons) as well as promote differentiation and repopulation by progenitor cells in the area affected. In preferred embodiments, a source of a *hedgehog* agent is stereotactically provided within or proximate the area of degeneration. In addition to degenerative-induced dementias, a pharmaceutical preparation of one or more of the subject *hedgehog* proteins can be applied opportunely in the treatment of neurodegenerative disorders which have manifestations of tremors and involuntary movements. Parkinson's disease, for example, primarily affects subcortical structures and is characterized by degeneration of the nigrostriatal pathway, raphe nuclei, locus cereleus, and the motor nucleus of vagus. Ballism is typically associated with damage to the subthalamic nucleus, often due to acute vascular accident. Also included are neurogenic and myopathic diseases which ultimately affect the somatic division of the peripheral nervous system and are manifest as neuromuscular disorders. Examples include chronic atrophies such as amyotrophic lateral sclerosis, Guillain-Barre syndrome and chronic peripheral neuropathy, as well as other diseases which can be manifest as progressive bulbar palsies or spinal muscular atrophies. The present method is amenable to the treatment of disorders of the cerebellum which result in hypotonia or ataxia, such as those lesions in the cerebellum which produce disorders in the limbs ipsilateral to the lesion. For instance, a preparation of a *hedgehog* homolog can be used to treat a restricted form of cerebellar cortical degeneration involving the anterior lobes (vermis and leg areas) such as is common in alcoholic patients.

In an illustrative embodiment, the subject method is used to treat amyotrophic lateral sclerosis. ALS is a name given to a complex of disorders that comprise upper and lower motor neurons. Patients may present with progressive spinal muscular atrophy, progressive bulbar palsy, primary lateral sclerosis, or a combination of these conditions. The major

pathological abnormality is characterized by a selective and progressive degeneration of the lower motor neurons in the spinal cord and the upper motor neurons in the cerebral cortex. The therapeutic application of a *hedgehog* agonist, particularly *Dhh*, can be used alone, or in conjunction with other neurotrophic factors such as CNTF, BDNF or NGF to prevent and/or reverse motor neuron degeneration in ALS patients.

Hedgehog proteins of the present invention can also be used in the treatment of autonomic disorders of the peripheral nervous system, which include disorders affecting the innervation of smooth muscle and endocrine tissue (such as glandular tissue). For instance, the subject method can be used to treat tachycardia or atrial cardiac arrhythmias which may arise from a degenerative condition of the nerves innervating the striated muscle of the heart.

Furthermore, a potential role for certain of the *hedgehog* proteins, which is apparent from the appended examples, mainly the data of respecting *hedgehog* expression in sensory and motor neurons of the head and trunk (including limb buds), concerns the role of *hedgehog* proteins in development and maintenance of dendritic processes of axonal neurons. Potential roles for *hedgehog* proteins consequently include guidance for axonal projections and the ability to promote differentiation and/or maintenance of the innervating cells to their axonal processes. Accordingly, compositions comprising *hedgehog* agonists or other *hedgehog* agents described herein, may be employed to support, or alternatively antagonize the survival and reprojction of several types of ganglionic neurons sympathetic and sensory neurons as well as motor neurons. In particular, such therapeutic compositions may be useful in treatments designed to rescue, for example, various neurons from lesion-induced death as well as guiding reprojction of these neurons after such damage. Such diseases include, but are not limited to, CNS trauma infarction, infection (such as viral infection with varicella-zoster), metabolic disease, nutritional deficiency, toxic agents (such as cisplatin treatment). Moreover, certain of the *hedgehog* agents (such as antagonistic form) may be useful in the selective ablation of sensory neurons, for example, in the treatment of chronic pain syndromes.

As appropriate, *hedgehog* agents can be used in nerve prostheses for the repair of central and peripheral nerve damage. In particular, where a crushed or severed axon is intubulated by use of a prosthetic device, *hedgehog* polypeptides can be added to the prosthetic device to increase the rate of growth and regeneration of the dendritic processes. Exemplary nerve guidance channels are described in U.S. patents 5,092,871 and 4,955,892. Accordingly, a severed axonal process can be directed toward the nerve ending from which it was severed by a prosthesis nerve guide which contains, e.g. a semi-solid formulation containing *hedgehog* polypeptide or mimetic, or which is derivatized along the inner walls with a *hedgehog* protein.

58

In another embodiment, the subject method can be used in the treatment of neoplastic or hyperplastic transformations such as may occur in the central nervous system. For instance, certain of the *hedgehog* proteins (or *hh* agonists) which induce differentiation of neuronal cells can be utilized to cause such transformed cells to become either post-mitotic or apoptotic. Treatment with a *hedgehog* agent may facilitate disruption of autocrine loops, such as TGF- β or PDGF autostimulatory loops, which are believed to be involved in the neoplastic transformation of several neuronal tumors. *Hedgehog* agonists may, therefore, be of use in the treatment of, for example, malignant gliomas, medulloblastomas, neuroectodermal tumors, and ependymomas.

Yet another aspect of the present invention concerns the application of the discovery that *hedgehog* proteins are morphogenic signals involved in other vertebrate organogenic pathways in addition to neuronal differentiation as described above, having apparent roles in other endodermal patterning, as well as both mesodermal and endodermal differentiation processes. As described in the Examples below, *Shh* clearly plays a role in proper limb growth and patterning by initiating expression of signaling molecules, including *Bmp-2* in the mesoderm and *Fgf-4* in the ectoderm. Thus, it is contemplated by the invention that compositions comprising *hedgehog* proteins can also be utilized for both cell culture and therapeutic methods involving generation and maintenance of non-neuronal tissue.

In one embodiment, the present invention makes use of the discovery that *hedgehog* proteins, such as *Shh*, are apparently involved in controlling the development of stem cells responsible for formation of the digestive tract, liver, lungs, and other organs which derive from the primitive gut. As described in the Examples below, *Shh* serves as an inductive signal from the endoderm to the mesoderm, which is critical to gut morphogenesis. Therefore, for example, *hedgehog* agonists can be employed in the development and maintenance of an artificial liver which can have multiple metabolic functions of a normal liver. In an exemplary embodiment, *hedgehog* agonists can be used to induce differentiation of digestive tube stem cells to form hepatocyte cultures which can be used to populate extracellular matrices, or which can be encapsulated in biocompatible polymers, to form both implantable and extracorporeal artificial livers.

In another embodiment, therapeutic compositions of *hedgehog* agonists can be utilized in conjunction with transplantation of such artificial livers, as well as embryonic liver structures, to promote intraperitoneal implantation, vascularization, and *in vivo* differentiation and maintenance of the engrafted liver tissue.

In yet another embodiment, *hedgehog* agonists can be employed therapeutically to regulate such organs after physical, chemical or pathological insult. For instance, therapeutic compositions comprising *hedgehog* agonists can be utilized in liver repair subsequent to a

partial hepatectomy. Similarly, therapeutic compositions containing *hedgehog* agonists can be used to promote regeneration of lung tissue in the treatment of emphysema.

In still another embodiment of the present invention, compositions comprising *hedgehog* agonists can be used in the *in vitro* generation of skeletal tissue, such as from skeletogenic stem cells, as well as the *in vivo* treatment of skeletal tissue deficiencies. The present invention particularly contemplates the use of *hedgehog* agonists which maintain a skeletogenic activity, such as an ability to induce chondrogenesis and/or osteogenesis. By "skeletal tissue deficiency", it is meant a deficiency in bone or other skeletal connective tissue at any site where it is desired to restore the bone or connective tissue, no matter how the deficiency originated, e.g. whether as a result of surgical intervention, removal of tumor, ulceration, implant, fracture, or other traumatic or degenerative conditions.

For instance, the present invention makes available effective therapeutic methods and compositions for restoring cartilage function to a connective tissue. Such methods are useful in, for example, the repair of defects or lesions in cartilage tissue which is the result of degenerative wear such as that which results in arthritis, as well as other mechanical derangements which may be caused by trauma to the tissue, such as a displacement of torn meniscus tissue, meniscectomy, a laxation of a joint by a torn ligament, malignment of joints, bone fracture, or by hereditary disease. The present reparative method is also useful for remodeling cartilage matrix, such as in plastic or reconstructive surgery, as well as periodontal surgery. The present method may also be applied to improving a previous reparative procedure, for example, following surgical repair of a meniscus, ligament, or cartilage. Furthermore, it may prevent the onset or exacerbation of degenerative disease if applied early enough after trauma.

In one embodiment of the present invention, the subject method comprises treating the afflicted connective tissue with a therapeutically sufficient amount of a *hedgehog* agonist, particularly an *Ihh* agonist, to generate a cartilage repair response in the connective tissue by stimulating the differentiation and/or proliferation of chondrocytes embedded in the tissue. Induction of chondrocytes by treatment with a *hedgehog* agonist can subsequently result in the synthesis of new cartilage matrix by the treated cells. Such connective tissues as articular cartilage, interarticular cartilage (menisci), costal cartilage (connecting the true ribs and the sternum), ligaments, and tendons are particularly amenable to treatment in reconstructive and/or regenerative therapies using the subject method. As used herein, regenerative therapies include treatment of degenerative states which have progressed to the point of which impairment of the tissue is obviously manifest, as well as preventive treatments of tissue where degeneration is in its earliest stages or imminent. The subject method can further be used to prevent the spread of mineralisation into fibrotic tissue by maintaining a constant production of new cartilage.

60

In an illustrative embodiment, the subject method can be used to treat cartilage of a diarthroidal joint, such as a knee, an ankle, an elbow, a hip, a wrist, a knuckle of either a finger or toe, or a temporomandibular joint. The treatment can be directed to the meniscus of the joint, to the articular cartilage of the joint, or both. To further illustrate, the subject method can be used to treat a degenerative disorder of a knee, such as which might be the result of traumatic injury (e.g., a sports injury or excessive wear) or osteoarthritis. An injection of a *hedgehog* agonist into the joint with, for instance, an arthroscopic needle, can be used to treat the afflicted cartilage. In some instances, the injected agent can be in the form of a hydrogel or other slow release vehicle described above in order to permit a more extended and regular contact of the agent with the treated tissue.

The present invention further contemplates the use of the subject method in the field of cartilage transplantation and prosthetic device therapies. To date, the growth of new cartilage from either transplantation of autologous or allogenic cartilage has been largely unsuccessful. Problems arise, for instance, because the characteristics of cartilage and fibrocartilage varies between different tissue: such as between articular, meniscal cartilage, ligaments, and tendons, between the two ends of the same ligament or tendon, and between the superficial and deep parts of the tissue. The zonal arrangement of these tissues may reflect a gradual change in mechanical properties, and failure occurs when implanted tissue, which has not differentiated under those conditions, lacks the ability to appropriately respond. For instance, when meniscal cartilage is used to repair anterior cruciate ligaments, the tissue undergoes a metaplasia to pure fibrous tissue. By promoting chondrogenesis, the subject method can be used to particularly addresses this problem, by causing the implanted cells to become more adaptive to the new environment and effectively resemble hypertrophic chondrocytes of an earlier developmental stage of the tissue. Thus, the action of chondrogenesis in the implanted tissue, as provided by the subject method, and the mechanical forces on the actively remodeling tissue can synergize to produce an improved implant more suitable for the new function to which it is to be put.

In similar fashion, the subject method can be applied to enhancing both the generation of prosthetic cartilage devices and to their implantation. The need for improved treatment has motivated research aimed at creating new cartilage that is based on collagen-glycosaminoglycan templates (Stone et al. (1990) *Clin Orthop Relat Res* 252:129), isolated chondrocytes (Grande et al. (1989) *J Orthop Res* 7:208; and Takigawa et al. (1987) *Bone Miner* 2:449), and chondrocytes attached to natural or synthetic polymers (Walitani et al. (1989) *J Bone Jt Surg* 71B:74; Vacanti et al. (1991) *Plast Reconstr Surg* 88:753; von Schroeder et al. (1991) *J Biomed Mater Res* 25:329; Freed et al. (1993) *J Biomed Mater Res* 27:11; and the Vacanti et al. U.S. Patent No. 5,041,138). For example, chondrocytes can be grown in culture on biodegradable, biocompatible highly porous scaffolds formed from

polymers such as polyglycolic acid, polylactic acid, agarose gel, or other polymers which degrade over time as function of hydrolysis of the polymer backbone into innocuous monomers. The matrices are designed to allow adequate nutrient and gas exchange to the cells until engraftment occurs. The cells can be cultured *in vitro* until adequate cell volume and density has developed for the cells to be implanted. One advantage of the matrices is that they can be cast or molded into a desired shape on an individual basis, so that the final product closely resembles the patient's own ear or nose (by way of example), or flexible matrices can be used which allow for manipulation at the time of implantation, as in a joint.

In one embodiment of the subject method, the implants are contacted with a *hedgehog* agonist during the culturing process, such as an *Ihh* agonist, in order to induce and/or maintain differentiated chondrocytes in the culture in order as to further stimulate cartilage matrix production within the implant. In such a manner, the cultured cells can be caused to maintain a phenotype typical of a chondrogenic cell (i.e. hypertrophic), and hence continue the population of the matrix and production of cartilage tissue.

In another embodiment, the implanted device is treated with a *hedgehog* agonist in order to actively remodel the implanted matrix and to make it more suitable for its intended function. As set out above with respect to tissue transplants, the artificial transplants suffer from the same deficiency of not being derived in a setting which is comparable to the actual mechanical environment in which the matrix is implanted. The activation of the chondrocytes in the matrix by the subject method can allow the implant to acquire characteristics similar to the tissue for which it is intended to replace.

In yet another embodiment, the subject method is used to enhance attachment of prosthetic devices. To illustrate, the subject method can be used in the implantation of a periodontal prosthesis, wherein the treatment of the surrounding connective tissue stimulates formation of periodontal ligament about the prosthesis, as well as inhibits formation of fibrotic tissue proximate the prosthetic device.

In still further embodiments, the subject method can be employed for the generation of bone (osteogenesis) at a site in the animal where such skeletal tissue is deficient. Indian *hedgehog* is particularly associated with the hypertrophic chondrocytes that are ultimately replaced by osteoblasts. For instance, administration of a *hedgehog* agent of the present invention can be employed as part of a method for treating bone loss in a subject, e.g. to prevent and/or reverse osteoporosis and other osteopenic disorders, as well as to regulate bone growth and maturation. For example, preparations comprising *hedgehog* agonists can be employed, for example, to induce endochondral ossification, at least so far as to facilitate the formation of cartilaginous tissue precursors to form the "model" for ossification. Therapeutic compositions of *hedgehog* agonists can be supplemented, if required, with other

osteoinductive factors, such as bone growth factors (e.g. TGF- β factors, such as the bone morphogenetic factors *BMP-2* and *BMP-4*, as well as activin), and may also include, or be administered in combination with, an inhibitor of bone resorption such as estrogen, bisphosphonate, sodium fluoride, calcitonin, or tamoxifen, or related compounds. However, it will be appreciated that *hedgehog* proteins, such as *Ihh* and *Shh* are likely to be upstream of BMPs, e.g. *hh* treatment will have the advantage of initiating endogenous expression of BMPs along with other factors.

In yet another embodiment of the present invention, a *hedgehog* antagonist can be used to inhibit spermatogenesis. Thus, in light of the present finding that *hedgehog* proteins are involved in the differentiation and/or proliferation and maintenance of testicular germ cells, *hedgehog* antagonist can be utilized to block the action of a naturally-occurring *hedgehog* protein. In a preferred embodiment, the *hedgehog* antagonist inhibits the biological activity of *Dhh* with respect to spermatogenesis, by competitively binding *hedgehog* receptors in the testis. In similar fashion, *hedgehog* agonists and antagonists are potentially useful for modulating normal ovarian function.

The source of *hedgehog* polypeptides, whether for cell culture or for *in vivo* application, can be in the form of a purified protein composition, or can be from a cell expressing either a recombinant or endogenous form of the polypeptide, such as embryonic tissue (e.g., floor plate tissue explants). Moreover, in addition to those forms of the vertebrate *hedgehog* polypeptides described herein, the present invention further contemplates the use of the *drosophila* *hedgehog* (Dros-HH) protein to induce cells and tissue of vertebrate organisms.

In the instance of protein compositions, the *hedgehog* protein, or a pharmaceutically acceptable salt thereof, may be conveniently formulated for administration with a biologically acceptable medium, such as water, buffered saline, polyol (for example, glycerol, propylene glycol, liquid polyethylene glycol and the like) or suitable mixtures thereof. The optimum concentration of the active ingredient(s) in the chosen medium can be determined empirically, according to procedures well known to medicinal chemists. As used herein, "biologically acceptable medium" includes any and all solvents, dispersion media, and the like which may be appropriate for the desired route of administration of the pharmaceutical preparation. The use of such media for pharmaceutically active substances is known in the art. Except insofar as any conventional media or agent is incompatible with the activity of the *hedgehog* protein, its use in the pharmaceutical preparation of the invention is contemplated. Suitable vehicles and their formulation inclusive of other proteins are described, for example, in the book *Remington's Pharmaceutical Sciences* (Remington's Pharmaceutical Sciences. Mack Publishing Company, Easton, Pa., USA 1985). These vehicles include injectable "deposit formulations". Based on the above, such pharmaceutical

63

formulations include, although not exclusively, solutions or freeze-dried powders of a *hedgehog* homolog (such as a *Shh*, *Dhh* or *Mhh*) in association with one or more pharmaceutically acceptable vehicles or diluents, and contained in buffered media at a suitable pH and isosmotic with physiological fluids. For illustrative purposes only and without being limited by the same, possible compositions or formulations which may be prepared in the form of solutions for the treatment of nervous system disorders with a *hedgehog* protein are given in U.S. Patent No. 5,218,094. In the case of freeze-dried preparations, supporting excipients such as, but not exclusively, mannitol or glycine may be used and appropriate buffered solutions of the desired volume will be provided so as to obtain adequate isotonic buffered solutions of the desired pH. Similar solutions may also be used for the pharmaceutical compositions of *hh* in isotonic solutions of the desired volume and include, but not exclusively, the use of buffered saline solutions with phosphate or citrate at suitable concentrations so as to obtain at all times isotonic pharmaceutical preparations of the desired pH, (for example, neutral pH).

Methods of introduction of exogenous *hh* at the site of treatment include, but are not limited to, intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous, oral, intranasal and topical. In addition, it may be desirable to introduce the pharmaceutical compositions of the invention into the central nervous system by any suitable route, including intraventricular and intrathecal injection. Intraventricular injection may be facilitated by an intraventricular catheter, for example, attached to a reservoir, such as an Ommaya reservoir.

Methods of introduction may also be provided by rechargeable or biodegradable devices. Various slow release polymeric devices have been developed and tested *in vivo* in recent years for the controlled delivery of drugs, including proteinacious biopharmaceuticals. A variety of biocompatible polymers (including hydrogels), including both biodegradable and non-degradable polymers, can be used to form an implant for the sustained release of an *hh* at a particular target site. Such embodiments of the present invention can be used for the delivery of an exogenously purified *hedgehog* protein, which has been incorporated in the polymeric device, or for the delivery of *hedgehog* produced by a cell encapsulated in the polymeric device.

An essential feature of certain embodiments of the implant can be the linear release of the *hh*, which can be achieved through the manipulation of the polymer composition and form. By choice of monomer composition or polymerization technique, the amount of water, porosity and consequent permeability characteristics can be controlled. The selection of the shape, size, polymer, and method for implantation can be determined on an individual basis according to the disorder to be treated and the individual patient response. The generation of such implants is generally known in the art. See, for example, *Concise Encyclopedia of Medical & Dental Materials*, ed. by David Williams (MIT Press: Cambridge, MA, 1990);

and the Sabel et al. U.S. Patent No. 4,883,666. In another embodiment of an implant, a source of cells producing a *hedgehog* protein, or a solution of hydrogel matrix containing purified *hh*, is encapsulated in implantable hollow fibers. Such fibers can be pre-spun and subsequently loaded with the *hedgehog* source (Aebischer et al. U.S. Patent No. 4,892,538; 5 Aebischer et al. U.S. Patent No. 5,106,627; Hoffman et al. (1990) *Expt. Neurobiol.* 110:39-44; Jaeger et al. (1990) *Prog. Brain Res.* 82:41-46; and Aebischer et al. (1991) *J. Biomech. Eng.* 113:178-183), or can be co-extruded with a polymer which acts to form a polymeric coat about the *hh* source (Lim U.S. Patent No. 4,391,909; Sefton U.S. Patent No. 4,353,888; Sugamori et al. (1989) *Trans. Am. Artif. Intern. Organs* 35:791-799; Sefton et al. (1987) 10 *Biotechnol. Bioeng.* 29:1135-1143; and Aebischer et al. (1991) *Biomaterials* 12:50-55).

In yet another embodiment of the present invention, the pharmaceutical *hedgehog* protein can be administered as part of a combinatorial therapy with other agents. For example, the combinatorial therapy can include a *hedgehog* protein with at least one trophic factor. Exemplary trophic factors include nerve growth factor, ciliary neurotrophic growth 15 factor, schwannoma-derived growth factor, glial growth factor, stiatal-derived neuronotrophic factor, platelet-derived growth factor, and scatter factor (HGF-SF). Antimitogenic agents can also be used, for example, when proliferation of surrounding glial cells or astrocytes is undesirable in the regeneration of nerve cells. Examples of such antimitotic agents include cytosine, arabinoside, 5-fluorouracil, hydroxyurea, and methotrexate.

20 Another aspect of the invention features transgenic non-human animals which express a heterologous *hedgehog* gene of the present invention, or which have had one or more genomic *hedgehog* genes disrupted in at least one of the tissue or cell-types of the animal. Accordingly, the invention features an animal model for developmental diseases, which animal has *hedgehog* allele which is mis-expressed. For example, a mouse can be bred which 25 has one or more *hh* alleles deleted or otherwise rendered inactive. Such a mouse model can then be used to study disorders arising from mis-expressed *hedgehog* genes, as well as for evaluating potential therapies for similar disorders.

Another aspect of the present invention concerns transgenic animals which are comprised of cells (of that animal) which contain a transgene of the present invention and 30 which preferably (though optionally) express an exogenous *hedgehog* protein in one or more cells in the animal. A *hedgehog* transgene can encode the wild-type form of the protein, or can encode homologs thereof, including both agonists and antagonists, as well as antisense constructs. In preferred embodiments, the expression of the transgene is restricted to specific subsets of cells, tissues or developmental stages utilizing, for example, cis-acting sequences 35 that control expression in the desired pattern. In the present invention, such mosaic expression of a *hedgehog* protein can be essential for many forms of lineage analysis and can additionally provide a means to assess the effects of, for example, lack of *hedgehog*

65

expression which might grossly alter development in small patches of tissue within an otherwise normal embryo. Toward this end, tissue-specific regulatory sequences and conditional regulatory sequences can be used to control expression of the transgene in certain spatial patterns. Moreover, temporal patterns of expression can be provided by, for example, conditional recombination systems or prokaryotic transcriptional regulatory sequences.

Genetic techniques which allow for the expression of transgenes can be regulated via site-specific genetic manipulation *in vivo* are known to those skilled in the art. For instance, genetic systems are available which allow for the regulated expression of a recombinase that catalyzes the genetic recombination a target sequence. As used herein, the phrase "target sequence" refers to a nucleotide sequence that is genetically recombined by a recombinase. The target sequence is flanked by recombinase recognition sequences and is generally either excised or inverted in cells expressing recombinase activity. Recombinase catalyzed recombination events can be designed such that recombination of the target sequence results in either the activation or repression of expression of one of the subject *hedgehog* proteins. For example, excision of a target sequence which interferes with the expression of a recombinant *hh* gene, such as one which encodes an antagonistic homolog or an antisense transcript, can be designed to activate expression of that gene. This interference with expression of the protein can result from a variety of mechanisms, such as spatial separation of the *hh* gene from the promoter element or an internal stop codon. Moreover, the transgene can be made wherein the coding sequence of the gene is flanked by recombinase recognition sequences and is initially transfected into cells in a 3' to 5' orientation with respect to the promoter element. In such an instance, inversion of the target sequence will reorient the subject gene by placing the 5' end of the coding sequence in an orientation with respect to the promoter element which allow for promoter driven transcriptional activation.

In an illustrative embodiment, either the *cre/loxP* recombinase system of bacteriophage P1 (Lakso et al. (1992) *PNAS* 89:6232-6236; Orban et al. (1992) *PNAS* 89:6861-6865) or the FLP recombinase system of *Saccharomyces cerevisiae* (O'Gorman et al. (1991) *Science* 251:1351-1355; PCT publication WO 92/15694) can be used to generate *in vivo* site-specific genetic recombination systems. Cre recombinase catalyzes the site-specific recombination of an intervening target sequence located between *loxP* sequences. *loxP* sequences are 34 base pair nucleotide repeat sequences to which the Cre recombinase binds and are required for Cre recombinase mediated genetic recombination. The orientation of *loxP* sequences determines whether the intervening target sequence is excised or inverted when Cre recombinase is present (Abremski et al. (1984) *J. Biol. Chem.* 259:1509-1514); catalyzing the excision of the target sequence when the *loxP* sequences are oriented as direct repeats and catalyzes inversion of the target sequence when *loxP* sequences are oriented as inverted repeats.

66

Accordingly, genetic recombination of the target sequence is dependent on expression of the Cre recombinase. Expression of the recombinase can be regulated by promoter elements which are subject to regulatory control, e.g., tissue-specific, developmental stage-specific, inducible or repressible by externally added agents. This regulated control will result in genetic recombination of the target sequence only in cells where recombinase expression is mediated by the promoter element. Thus, the activation expression of a recombinant *hedgehog* protein can be regulated via control of recombinase expression.

Use of the *cre/loxP* recombinase system to regulate expression of a recombinant *hh* protein requires the construction of a transgenic animal containing transgenes encoding both the Cre recombinase and the subject protein. Animals containing both the Cre recombinase and a recombinant *hedgehog* gene can be provided through the construction of "double" transgenic animals. A convenient method for providing such animals is to mate two transgenic animals each containing a transgene, e.g., an *hh* gene and recombinase gene.

One advantage derived from initially constructing transgenic animals containing a *hedgehog* transgene in a recombinase-mediated expressible format derives from the likelihood that the subject protein, whether agonistic or antagonistic, can be deleterious upon expression in the transgenic animal. In such an instance, a founder population, in which the subject transgene is silent in all tissues, can be propagated and maintained. Individuals of this founder population can be crossed with animals expressing the recombinase in, for example, one or more tissues and/or a desired temporal pattern. Thus, the creation of a founder population in which, for example, an antagonistic *hh* transgene is silent will allow the study of progeny from that founder in which disruption of *hedgehog* mediated induction in a particular tissue or at certain developmental stages would result in, for example, a lethal phenotype.

Similar conditional transgenes can be provided using prokaryotic promoter sequences which require prokaryotic proteins to be simultaneously expressed in order to facilitate expression of the *hedgehog* transgene. Exemplary promoters and the corresponding trans-activating prokaryotic proteins are given in U.S. Patent No. 4,833,080.

Moreover, expression of the conditional transgenes can be induced by gene therapy-like methods wherein a gene encoding the trans-activating protein, e.g. a recombinase or a prokaryotic protein, is delivered to the tissue and caused to be expressed, such as in a cell-type specific manner. By this method, a *hedgehog* transgene could remain silent into adulthood until "turned on" by the introduction of the trans-activator.

In an exemplary embodiment, the "transgenic non-human animals" of the invention are produced by introducing transgenes into the germline of the non-human animal. Embryonic target cells at various developmental stages can be used to introduce transgenes.

Different methods are used depending on the stage of development of the embryonic target cell. The zygote is the best target for micro-injection. In the mouse, the male pronucleus reaches the size of approximately 20 micrometers in diameter which allows reproducible injection of 1-2pl of DNA solution. The use of zygotes as a target for gene transfer has a major advantage in that in most cases the injected DNA will be incorporated into the host gene before the first cleavage (Brinster et al. (1985) *PNAS* 82:4438-4442). As a consequence, all cells of the transgenic non-human animal will carry the incorporated transgene. This will in general also be reflected in the efficient transmission of the transgene to offspring of the founder since 50% of the germ cells will harbor the transgene. Microinjection of zygotes is the preferred method for incorporating transgenes in practicing the invention.

Retroviral infection can also be used to introduce *hedgehog* transgenes into a non-human animal. The developing non-human embryo can be cultured *in vitro* to the blastocyst stage. During this time, the blastomeres can be targets for retroviral infection (Jaenich, R. (1976) *PNAS* 73:1260-1264). Efficient infection of the blastomeres is obtained by enzymatic treatment to remove the zona pellucida (*Manipulating the Mouse Embryo*, Hogan eds. (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, 1986). The viral vector system used to introduce the transgene is typically a replication-defective retrovirus carrying the transgene (Jahner et al. (1985) *PNAS* 82:6927-6931; Van der Putten et al. (1985) *PNAS* 82:6148-6152). Transfection is easily and efficiently obtained by culturing the blastomeres on a monolayer of virus-producing cells (Van der Putten, *supra*; Stewart et al. (1987) *EMBO J.* 6:383-388). Alternatively, infection can be performed at a later stage. Virus or virus-producing cells can be injected into the blastocoele (Jahner et al. (1982) *Nature* 298:623-628). Most of the founders will be mosaic for the transgene since incorporation occurs only in a subset of the cells which formed the transgenic non-human animal. Further, the founder may contain various retroviral insertions of the transgene at different positions in the genome which generally will segregate in the offspring. In addition, it is also possible to introduce transgenes into the germ line by intrauterine retroviral infection of the midgestation embryo (Jahner et al. (1982) *supra*).

A third type of target cell for transgene introduction is the embryonic stem cell (ES). ES cells are obtained from pre-implantation embryos cultured *in vitro* and fused with embryos (Evans et al. (1981) *Nature* 292:154-156; Bradley et al. (1984) *Nature* 309:255-258; Gossler et al. (1986) *PNAS* 83: 9065-9069; and Robertson et al. (1986) *Nature* 322:445-448). Transgenes can be efficiently introduced into the ES cells by DNA transfection or by retrovirus-mediated transduction. Such transformed ES cells can thereafter be combined with blastocysts from a non-human animal. The ES cells thereafter colonize the embryo and contribute to the germ line of the resulting chimeric animal. For review see Jaenisch, R. (1988) *Science* 240:1468-1474.

68

Methods of making *hedgehog* knock-out or disruption transgenic animals are also generally known. See, for example, *Manipulating the Mouse Embryo*, (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1986). Recombinase dependent knockouts can also be generated, e.g. by homologous recombination to insert recombinase target sequences flanking portions of an endogenous *hh* gene, such that tissue specific and/or temporal control of inactivation of a *hedgehog* allele can be controlled as above.

Exemplification

The invention, now being generally described, will be more readily understood by reference to the following examples, which are included merely for purposes of illustration of certain aspects and embodiments of the present invention and are not intended to limit the invention.

Example 1

Cloning and Expression of Chick Sonic Hedgehog

(i) Experimental Procedures

Using degenerate PCR primers, vHH5O (SEQ ID No:18), vHH3O (SEQ ID No:19) and vHH3I (SEQ ID No:20) corresponding to a sequence conserved between *Drosophila hedgehog* (Dros-HH)(SEQ ID No:34) (Lee, J.J. et al. (1992) *Cell* 71: 33-50; Mohler, J. et al., (1992) *Development* 115: 957-971) and mouse Indian *hedgehog* (*Ihh*) (SEQ ID No:10), a 220 base pair (bp) fragment was amplified from chicken genomic DNA. From 15 isolates, two distinct sequences were cloned, pCHA (SEQ ID No:35) and pCHB (SEQ ID No:36), each highly homologous to mouse *Ihh* (Figure 1). A probe made from isolate pCHA did not detect expression in embryonic tissues. Isolate pCHB, however, detected a 4 kb message in RNA prepared from embryonic head, trunk, or limb bud RNA. This cloned PCR fragment was therefore used as a probe to screen an unamplified cDNA library prepared from Hamburger Hamilton stage 22 (Hamburger, W. et al., (1951) *J. Morph.* 88: 49-92) limb bud RNA as described below.

A single 1.6 kilobase (kb) cDNA clone, pHH-2, was selected for characterization and was used in all subsequent analyses. The gene encoding for this cDNA was named *Sonic Hedgehog* (after the Sega computer game cartoon character). Sequencing of the entire cDNA confirmed the presence of a single long open reading frame potentially encoding for a protein of 425 amino acids (aa). The clone extends 220 bp upstream of the predicted initiator methionine and approximately 70 bp beyond the stop codon. No consensus polyadenylation signal could be identified in the 3' untranslated region. A second potential initiator

69

methionine occurs at amino acid residue 4. The putative translation initiation signals surrounding both methionines are predicted to be equally efficient (Kozak, M., (1987) *Nuc. Acids Res.* 15: 8125-8132). When the pHH-2 *Sonic* cDNA is used to probe a northern blot of stage 24 embryonic chick RNA, a single mRNA species of approximately 4 kb is detected in both limb and trunk tissue. The message size was predicted by comparing it to the position of 18S and 28S ribosomal RNA. Hybridized mRNA was visualized after a two day exposure to a phosphoscreen. Because the *Sonic* cDNA clone pHH-2 is only 1.6 kb, it is likely to be missing approximately 2.4 kb of untranslated sequence.

PCR Cloning

All standard cloning techniques were performed according to Ausubel et. al. (1989), and all enzymes were obtained from Boehringer Mannheim Biochemicals. Degenerate oligonucleotides corresponding to amino acid residues 161 to 237 of the *Drosophila hedgehog* protein (SEQ ID No:34) (Lee, J.J. et. al., (1992) *Cell* 71: 33-50) were synthesized. These degenerate oligonucleotides, vHH5O (SEQ ID No:18), vHH3O (SEQ ID No:19), and vHH3I (SEQ ID No:20) also contained Eco RI, Cla I, and Xba I sites, respectively, on their 5' ends to facilitate subcloning. The nucleotide sequence of these oligos is given below:

vHH5O: 5'-GGAATTCCCAG(CA)GITG(CT)AA(AG)GA(AG)(CA)(AG)I(GCT)IAA-3'
 vHH3O: 5'-TCATCGATGGACCCA(GA)TC(GA)AAICCGC(TC)TC-3'
 vHH3I: 5'-GCTCTAGAGCTCIACIGCIA(GA)IC(GT)IGC-3'

where I represents inosine. Nested PCR was performed by first amplifying chicken genomic DNA using the vHH5O and vHH3O primer pair and then further amplifying that product using the vHH5O and vHH3I primer pair. In each case the reaction conditions were: initial denaturation at 93° C for 2.5 min., followed by 30 cycles of 94° C for 45 s, 50° C for 1 min., 72° C for 1, and a final incubation of 72° C for 5 min. The 220 bp PCR product was subcloned into pGEM7zf (Promega). Two unique clones, pCHA (SEQ ID No:35) and pCHB (SEQ ID No:36) were identified.

DNA Sequence Analysis

Nucleotide sequences were determined by the dideoxy chain termination method (Sanger, F. et al., (1977) *Proc. Natl. Acad. Sci. USA* 74: 5463-5467) using Sequenase v2.0 T7 DNA polymerase (US Biochemicals). 5' and 3' nested deletions of pHH-2 were generated by using the nucleases Exo III and S1 (Erase a Base, Promega) and individual subclones sequenced. DNA and amino acid sequences were analyzed using both GCG (Devereux, J. et al., (1984) *Nuc. Acids Res.* 12: 387-394) and DNASTAR software. Searches for related

sequences were done through the BLAST network service (Altschul, S.F. et al., (1990) *J. Mol. Biol.* 215: 403-410) provided by the National Center for Biotechnology Information.

Southern Blot Analysis

Five (5) µg of chick genomic DNA was digested with Eco RI and/or Bam HI, fractionated on a 1% agarose gel, and transferred to a nylon membrane (Genescreen, New England Nuclear). The filters were probed with ³²P-labeled *hha* or *hhb* at 42°C in hybridization buffer (0.5% BSA, 500 mM NaHPO₄, 7% SDS, 1 mM EDTA, pH 7.2; Church, G.M. et al., (1984) *Proc. Natl. Acad. Sci. USA* 81: 1991-1995). The blots were washed at 63°C once in 0.5% bovine serum albumin, 50 mM NaHPO₄ (pH 7.2), 5% SDS, 1 mM EDTA and twice in 40 mM NaHPO₄ (pH 7.2), 1% SDS, 1mM EDTA, and visualized on Kodak XAR-5 film.

Isolation Of Chicken Sonic cDNA Clones

A stage 22 limb bud cDNA library was constructed in λgt10 using Eco RI/NotI linkers. Unamplified phage plaques (10⁶) were transferred to nylon filters (Colony/Plaque screen, NEN) and screened with α³²P-labelled pooled inserts from PCR clones pCHA (SEQ ID No:35) and pCHB (SEQ ID No:36). Hybridization was performed at 42° C in 50% formamide 2X SSC, 10% dextran sulfate, 1% SDS and washing as described in the Southern Blot procedure. Eight positive plaques were identified, purified and their cDNA inserts excised with EcoRI and subcloned into pBluescript SK+ (Stratagene). All eight had approximately 1.7 kb inserts with identical restriction patterns. One, pHH-2, was chosen for sequencing and used in all further manipulations.

Preparation Of Digoxigenin-Labeled Riboprobes

Plasmid pHH-2 was linearized with Hind III and transcribed with T3 RNA polymerase (for antisense probes) or with Bam HI and transcribed with T7 RNA polymerase according to the manufacturers instructions for the preparation of non-radioactive digoxigenin transcripts. Following the transcription reaction, RNA was precipitated, and resuspended in RNase-free water.

Whole Mount In Situ Hybridization

Whole-mount *in situ* hybridization was performed using protocols modified from Parr, B.A. et al.. (1993) *Development* 119: 247-261; Sasaki, H. et al. (1993) *Development* 118: 47-59; Rosen, B. et al. (1993) *Trends Genet.* 9: 162-167. Embryos from incubated fertile White Leghorn eggs (Spafas) were removed from the egg and extra-embryonic membranes dissected in calcium/magnesium-free phosphate-buffered saline (PBS) at room temperature. Unless otherwise noted, all washes are for five minutes at room temperature.

Embryos were fixed overnight at 4°C with 4% paraformaldehyde in PBS, washed twice with PBT (PBS with 0.1% Tween-20) at 4°C, and dehydrated through an ascending methanol series in PBT (25%, 50%, 75%, 2 X 100% methanol). Embryos were stored at -20°C until further use.

5 Both pre-limb bud and limb bud stage embryos were rehydrated through an descending methanol series followed by two washes in PBT. Limb bud stage embryos were bleached in 6% hydrogen peroxide in PBT, washed three times with PBT, permeabilized with proteinase K (Boehringer, 2 µg/ml) for 15 minutes, washed with 2 mg/ml glycine in PBT for 10 minutes, and twice with PBT. Pre-limb bud stage embryos were permeabilized (without
10 prior incubation with hydrogen peroxide) by three 30 minute washes in RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% Deoxycholate, 0.1% SDS, 1mM EDTA, 50 mM Tris-HCl, pH 8.0). In all subsequent steps, pre-limb bud and limb bud stage embryos were treated equivalently. Embryos were fixed with 4% paraformaldehyde/0.2% glutaraldehyde in PBT, washed four times with PBT, once with pre-hybridization buffer (50% formamide, 5 X SSC,
15 1% SDS, 50 µg/ml total yeast RNA, 50 µg/ml heparin, pH 4.5), and incubated with fresh pre-hybridization buffer for one hour at 70°C. The pre-hybridization buffer was then replaced with hybridization buffer (pre-hybridization buffer with digoxigenin labeled riboprobe at 1 µg/ml) and incubated overnight at 70°C.

Following hybridization, embryos were washed 3 X 30 minutes at 70°C with solution
20 1 (50% formamide, 5 X SSC, 1% SDS, pH 4.5), 3 X 30 minutes at 70°C with solution 3 (50% formamide, 2 X SSC, pH 4.5), and three times at room temperature with TBS (Tris-buffered saline with 2 mM levamisole) containing 0.1% Tween-20. Non-specific binding of antibody was prevented by preblocking embryos in TBS/0.1% Tween-20 containing 10% heat-inactivated sheep serum for 2.5 hours at room temperature and by pre-incubating anti-digoxigenin Fab alkaline-phosphatase conjugate (Boehringer) in TBS/0.1% Tween-20
25 containing heat inactivated 1% sheep serum and approximately 0.3% heat inactivated chick embryo powder. After an overnight incubation at 4°C with the pre-adsorbed antibody in TBS/0.1% Tween-20 containing 1% sheep serum, embryos were washed 3 X 5 minutes at room temperature with TBS/0.1% Tween-20, 5 X 1.5 hour room temperature washes with
30 TBS/1% Tween-20, and overnight with TBS/1% Tween-20 at 4°C. The buffer was exchanged by washing 3 X 10 minutes with NTMT (100mM NaCl, 100 mM Tris-HCl, 50 mM MgCl₂, 0.1% Tween-20, 2 mM levamisole). The antibody detection reaction was performed by incubating embryos with detection solution (NTMT with 0.25 mg/ml NBT and 0.13 mg/ml X-Phos). In general, pre-limb bud stage embryos were incubated for 5-15 hours
35 and limb bud stage embryos 1-5 hours. After the detection reaction was deemed complete, embryos were washed twice with NTMT, once with PBT (pH 5.5), postfixed with 4% paraformaldehyde/0.1% glutaraldehyde in PBT, and washed several times with PBT. In

some cases embryos were cleared through a series of 30%, 50%, 70%, and 80% glycerol in PBT. Whole embryos were photographed under transmitted light using a Nikon zoom stereo microscope with Kodak Ektar 100 ASA film. Selected embryos were processed for frozen sections by dehydration in 30% sucrose in PBS followed by embedding in gelatin and freezing. 25 µm cryostat sections were collected on superfrost plus slides (Fisher), rehydrated in PBS, and mounted with gelvatol. Sections were photographed with Nomarski optics using a Zeiss Axiophot microscope and Kodak Ektar 25 ASA film.

(ii) *Sequence Homology Comparison Between Chicken Sonic hh And Dros-HH And Other Vertebrate Sonic hh Proteins*

The deduced *Sonic* amino acid sequence (SEQ ID No:8) is shown and compared to the *Drosophila hedgehog* protein (SEQ ID No:34) in Figure 2. Over the entire open reading frame the two proteins are 48% homologous at the amino acids level. The predicted *Drosophila* protein extends 62 aa beyond that of *Sonic* at its amino terminus. This N-terminal extension precedes the putative signal peptide (residues 1-26) of the fly protein (SEQ ID No:34), and has been postulated to be removed during processing of the secreted form of *Drosophila hedgehog* (Lee, J.J. et al., (1992) *Cell* 71: 33-50). The sequence of residues 1-26 of the *Sonic* protein (SEQ ID No:8) matches well with consensus sequences for eukaryotic signal peptides (Landry, S.J. et al., (1993) *Trends. Biochem. Sci.* 16: 159-163) and is therefore likely to serve that function for *Sonic*. Furthermore, Figure 3 shows a hydropathy plot (Kyte, J. et al., (1982) *J. Mol. Biol.* 157: 133-148) indicating that residues 1-26 of the *Sonic* protein (SEQ ID No:8) exhibit a high hydrophobic moment in accord with identified eukaryotic signal peptides. Cleavage of the putative signal sequence should occur C-terminal to residue 26 according to the predictive method of von Henjje, G. (1986) *Nucl. Acid. Res.* 11: 1986. A single potential N-linked glycosylation site is located at amino acid residue 282 of the *Sonic* protein (SEQ ID No:8). The predicted *Sonic* protein does not contain any other strong consensus motifs, and is not homologous to any other proteins outside of the *Hedgehog* family.

The mouse (SEQ ID No:11) and zebrafish (SEQ ID No:12) homologs of *Sonic* have also been isolated. A comparison of these and the *Drosophila* sequence is shown schematically in Figure 4. All of the vertebrate proteins have a similar predicted structure: a putative signal peptide at their amino terminus, followed by an extraordinarily similar 182 amino acid region (99% identity in chicken versus mouse and 95% identity in chicken versus zebrafish) and a less well conserved carboxy-terminal region.

(iii) *At Least Three Hedgehog Homologues Are Present In The Chicken Genome*

Since two distinct PCR products encoding for chicken *hedgehogs* were amplified from genomic DNA, the total number of genes in the chicken *hedgehog* family needed to be

estimated. The two PCR clones pCHA (SEQ ID No:35) and pCHB (SEQ ID No:36) were used to probe a genomic Southern blot under moderately stringent conditions as described in the above Experimental Procedures. The blot was generated by digesting 5 µg of chick chromosomal DNA with EcoRI and BamHI alone and together. Each probe reacted most strongly with a distinct restriction fragment. For example, the blot probed with pCHA, shows three bands in each of the Bam HI lanes, one strong at 6.6 kb and two weak at 3.4 and 2.7 kb. The blot probed with pCHB, shows the 2.7 kb band as the most intense, while the 3.4 and 6.6 kb bands are weaker. A similar variation of intensities can also be seen in the Bam HI/Eco RI and EcoRI lanes. Exposure times were 72 hr. This data indicates that each probe recognizes a distinct chicken *hedgehog* gene, and that a third as yet uncharacterized chicken *hedgehog* homolog exists in the chicken genome.

(iv) *Northern Analysis Defining Sites Of Sonic Transcription*

Northern analysis was performed which confirmed that *Sonic* is expressed during chick development. The spatial and temporal expression of *Sonic* in the chick embryo from gastrulation to early organogenesis was determined by whole mount *in situ* hybridization using a riboprobe corresponding to the full-length *Sonic* cDNA (SEQ ID No:1).

20µg total RNA isolated from stage 24 chick leg buds or bodies (without heads or limbs) was fractionated on a 0.8% agarose formaldehyde gel and transferred to a nylon membrane (Hybond N, Amersham). The blot was probed with the 1.6 kb EcoRI insert from pHH-2. Random-primed $\alpha^{32}\text{P}$ -labelled insert was hybridized at 42°C hybridization buffer (1% BSA, 500mM NaHPO₄, 7% SDS, 1 mM EDTA, pH 7.2) and washed at 63° C once in 0.5% bovine serum albumin, 50 mM NaHPO₄ (pH 7.2), 5% SDS, 1 mM EDTA and once in 40 mM NaHPO₄ (pH 7.2), 1% SDS, 1mM EDTA. The image was visualized using a phosphoimager (Molecular Dynamics) and photographed directly from the video monitor.

(v) *Expression Of Sonic During Mid-Gastrulation*

Sonic message is detected in the gastrulating blastoderm at early stage 4, the earliest stage analyzed. Staining is localized to the anterior end of the primitive streak in a region corresponding to Hensen's node. As gastrulation proceeds, the primitive streak elongates to its maximal cranial-caudal extent, after which Hensen's node regresses caudally and the primitive streak shortens. At an early point of node regression, *Sonic* mRNA can be detected at the node and in midline cells anterior to the node. By late stage 5, when the node has migrated approximately one-third of the length of the fully elongated primitive streak, prominent *Sonic* expression is seen at the node and in the midline of the embryo, reaching its anterior limit at the developing head process. Sections at a cranial level show that *Sonic* mRNA is confined to invaginated axial mesendoderm, tissue which contributes to foregut and notochord. More caudally, but still anterior to Hensen's node, staining of axial

74

mesoderm is absent and *Sonic* expression is confined to the epiblast. At the node itself, high levels of *Sonic* message are observed in an asymmetric distribution extending to the left of and posterior to the primitive pit. This asymmetric distribution is consistently observed (6/6 embryos from stages 5-7) and is always located to the left of the primitive pit. At the node, and just posterior to the node, *Sonic* expression is restricted to the epiblast and is not observed in either mesoderm or endoderm. The expression of *Sonic* in the dorsal epiblast layer without expression in underlying axial mesoderm contrasts markedly with later stages where *Sonic* expression in underlying mesoderm always precedes midline neural tube expression.

(vi) *Expression Of Sonic During Head Fold Stages*

During the formation and differentiation of the head process, *Sonic* mRNA is detected in midline cells of the neural tube, the foregut, and throughout most of the axial mesoderm. At stage 7, *Sonic* message is readily detected asymmetrically at the node and in ventral midline cells anterior to the node. The rostral limit of *Sonic* expression extends to the anterior-most portions of the embryo where it is expressed in the foregut and prechordal mesoderm (Adelmann, H.B., (1932) *Am. J. Anat.* 31, 55-101). At stage 8, expression of *Sonic* persists along the entire ventral midline anterior to Hensen's node, while the node region itself no longer expresses *Sonic*. Transverse sections at different axial levels reveal that at stage 8 *Sonic* is coexpressed in the notochord and the overlying ventromedial neuroectoderm from anterior to Hensen's node to the posterior foregut. The levels of *Sonic* message are not uniform in the neural tube: highest levels are found at the presumptive mid- and hindbrain regions with progressively lower levels anterior and posterior. The increasing graded expression in the neural tube from Hensen's node to the rostral brain may reflect the developmental age of the neuroectoderm as differentiation proceeds from posterior to anterior. At the anterior-most end of the embryo, expression is observed in midline cells of the dorsal and ventral foregut as well as in prechordal mesoderm. Although the prechordal mesoderm is in intimate contact with the overlying ectoderm, the latter is devoid of *Sonic* expression.

(vii) *Expression Of Sonic During Early CNS Differentiation*

At stages 10 through 14, *Sonic* expression is detected in the notochord, ventral neural tube (including the floor plate), and gut precursors. By stage 10, there is a marked expansion of the cephalic neuroectoderm, giving rise to the fore- mid- and hind-brain. At stage 10, *Sonic* mRNA is abundantly expressed in the ventral midline of the hindbrain and posterior midbrain. This expression expands laterally in the anterior midbrain and posterior forebrain. Expression does not extend to the rostral forebrain at this or later stages. Sections reveal that *Sonic* is expressed in the notochord, the prechordal mesoderm, and the anterior midline of the

foregut. Expression in the neuroepithelium extends from the forebrain caudally. In the posterior-most regions of the embryo which express *Sonic*, staining is found only in the notochord and not in the overlying neurectoderm. This contrasts with earlier expression in which the posterior domains of *Sonic* expression contain cells are located in the dorsal epiblast, but not in underlying mesoderm or endoderm. Midgut precursors at the level of the anterior intestinal portal also show weak *Sonic* expression.

At stage 14, expression continues in all three germ layers. The epithelium of the closing midgut expresses *Sonic* along with portions of the pharyngeal endoderm and anterior foregut. Ectoderm lateral and posterior to the tail bud also exhibits weak expression. At this stage, *Sonic* is also expressed along entire length of the notochord which now extends rostrally only to the midbrain region and no longer contacts the neuroepithelium at the anterior end of the embryo. Expression in head mesenchyme anterior to the notochord is no longer observed. In the neural tube *Sonic* is found along the ventral midline of the fore- mid- and hindbrain and posteriorly in the spinal cord. In the forebrain, expression is expanded laterally relative to the hindbrain. At midgut levels, expression of *Sonic* in the neural tube appears to extend beyond the floor plate into more lateral regions. As observed at stage 10, *Sonic* at stage 14 is found in the notochord, but not in the ventral neural tube in posterior-most regions of the embryo. When neuroectodermal expression is first observed in the posterior embryo, it is located in midline cells which appear to be in contact with the notochord. At later stages, expression continues in areas which show expression at stage 14, namely the CNS, gut epithelium including the allantoic stalk, and axial mesoderm.

(viii) Sonic Is Expressed In Posterior Limb Bud Mesenchyme

The limb buds initially form as local thickenings of the lateral plate mesoderm. As distal outgrowth occurs during stage 17, *Sonic* expression becomes apparent in posterior regions of both the forelimb and the hindlimb. Sections through a stage 21 embryo at the level of the forelimbs reveal that expression of *Sonic* in limb buds is limited to mesenchymal tissue. A more detailed expression profile of *Sonic* during limb development is discussed below in Example 3. Briefly, as the limb bud grows out, expression of *Sonic* narrows along the anterior-posterior axis to become a thin stripe along the posterior margin closely apposed to the ectoderm. Expression is not found at more proximal regions of the bud. High levels of *Sonic* expression are maintained until around stage 25/26 when staining becomes weaker. Expression of *Sonic* is no longer observed in wing buds or leg buds after stage 28.

76
Example 2

Mouse Sonic Hedgehog Is Implicated in the Regulation of CNS and Limb Polarity

(i) *Experimental Procedures*

Isolation Of Hedgehog Phage Clones

5 The initial screen for mammalian *hh* genes was performed, as above, using a 700bp PCR fragment encompassing exons 1 and 2 of the Dros-HH gene. Approximately one million plaques of a 129/Sv Lambda Fix II genomic library (Stratagene) were hybridized with an α ^{32}P -dATP labeled probe at low stringency (55°C in 6xSSC, 0.5%SDS, 5 x Denhardt's; final wash at 60°C in 0.5 x SSC, 0.1% SDS for 20'). Five cross hybridizing
10 phage plaques corresponding to the *Dhh* gene were purified. Restriction enzyme analysis indicated that all clones were overlapping. Selected restriction enzyme digests were then performed to map and subclone one of these. Subclones in pGEM (Promega) or Bluescript (Stratagene) which cross-hybridized with the Dros-HH fragment where sequenced using an ABI automatic DNA sequencer.

15 Mouse *Ihh* and *Shh* were identified by low stringency hybridization (as described above) with a chick *Shh* cDNA clone to one million plaques of an 8.5 day λ gt10 mouse embryo cDNA library (Fahrner, K. et al., (1987) *EMBO J.* 6: 1265-1271). Phage plaques containing a 1.8kb *Ihh* and 0.64 and 2.8kb *Shh* inserts were identified. Inserts were excised and subcloned into Bluescript (Stratagene) for dideoxy chain termination sequencing using
20 modified T7 DNA polymerase (USB). The larger *Shh* clone contained a partially processed cDNA in which intron splicing at the exon 1/2 junction had not occurred.

 To screen for additional *Ihh* and *Shh* cDNA clones, an 8.5 day λ ZAPII cDNA library was probed at high stringency (at 65°C in 6xSSC, 0.5% SDS, 5 x Denhardt's; final wash at 65 °C in 0.1xSSC, 0.1% SDS for 30') with the *Ihh* and *Shh* mouse cDNA clones. No additional
25 *Ihh* clones were identified. However several 2.6kb, apparently full length, *Shh* clones were isolated. The DNA sequence of the additional 5' coding region not present in the original 0.64 and 2.8kb *Shh* clones was obtained by analysis of one of the 2.6kb inserts.

Northern Blot Analysis

 Expression of *Shh* was investigated by RNA blot analysis using 20 μg of total RNA
30 from adult brain, spleen, kidney, liver, lung, 16.5dpc brain, liver and lung; 9.5dpc to 17.5dpc whole embryo; 9.5dpc forebrain, midbrain and 10.5dpc brain. RNA samples were electrophoretically separated on a 1.2% agarose gel, transferred and u.v. crosslinked to Genescreen (DuPont) and probed with 2×10^6 cpm/ml of an α ^{32}P -dATP labeled mouse *Shh* probe (2.8kb insert from λ gt 10 screen). Hybridization was performed at 42°C in 50%
35 formamide 5x Denhardt's, 5xSSPE, 0.1%SDS, 6.5% dextran, 200 μg /ml salmon sperm DNA.

Final wash was at 55°C in 0.1xSSC, 0.1%SDS. The blot was exposed for 6 days in the presence of an intensifying screen.

In Situ Hybridization, β -Galactosidase Staining And Histological Analysis

Embryos from 7.25 to 14.5dpc were analyzed for either *Shh* or HNF-3 β expression by whole mount *in situ* hybridization to digoxigenin labeled RNA probes as described in Wilkinson, (1992) *In situ Hybridization: A Practical Approach*. Oxford; Parr et al., (1993) *Development* 119:247-261. The mouse *Shh* probe was either a 2.8kb or 0.6kb RNA transcript generated by T7 (2.8kb) or T3 (0.6kb) transcription of XbaI and HindIII digests of Bluescript (Stratagene) subclones of the original *Shh* cDNA inserts. The HNF-3 β probe was generated by HindIII linearization of a HNF-3 β cDNA clone (Sasaki, H. et al., (1993) *Development* 118: 47-59) and T7 polymerase transcription of 1.6kb transcript. Embryos were photographed on an Olympus-SZH photomicroscope using Kodak Ektachrome EPY 64T color slide film.

Sections through wild type and WEXP2-C*Shh* transgenic embryos were prepared and hybridized with ³⁵S-UIP labeled RNA probes (Wilkinson, D.G. et al., (1987) *Development* 99: 493-500). Sections were photographed as described in McMahon, A.P. et al., (1992) *Cell* 69: 581-595.

β Staining of WEXP2-lacZ embryos with β was performed according to Whiting, J. et al., (1991) *Genes & Dev.* 5: 2048-2059. General histological analysis of wildtype and WEXP2-C*Shh* transgenic embryos was performed on paraffin sections of Bouin's fixed embryos counterstained with hematoxylin and eosin. Histological procedures were as described by Kaufman, M.H. (1992) *The Atlas of Mouse Development*, London: Academic Press. Sections were photographed on a Leitz Aristoplan compound microscope using Kodak EPY 64T color slide film.

DNA Constructs For Transgenics

Genomic *Wnt-1* fragments were obtained by screening a λ GEM12 (Promega) 129/Sv mouse genomic library with a 375 bp *MluI*-*BglII* fragment derived from the fourth exon of the murine *Wnt-1* gene. One of the clones (W1-15.1) was used in this study.

As an initial step towards the generation of the pWEXP2 expression vector, W1-15.1 was digested to completion with restriction enzymes *AatII* and *ClaI*, and a 2774 bp *AatII*-*ClaI* fragment isolated. This fragment was ligated into *AatII* and *ClaI* cut pGEM-7Zf vector (Promega), generating pW1-18. This plasmid was digested with *HindII* and ligated to annealed oligonucleotides *lacI* (SEQ ID No:21) and *lac2* (SEQ ID No:22) generating pW1-18S* which has a modified polylinker downstream of the *ClaI* restriction site. This construct (pW1-18S*) was digested with *ClaI* and *BglII* and ligated with both the 2.5 kb 3' *ClaI* - *BglII*

78

exon-intron region and 5.5 kb 3' *Bgl*III -*Bgl*III *Wnt-1* enhancer, generating pWRES4. This construct contains a 10.5 kb genomic region which starts upstream of the *Wnt-1* translation initiation codon (at an *Aat*II site approximately 1.0kb from the ATG) and extends to a *Bgl*III site 5.5 kb downstream of the *Wnt-1* polyadenylation signal. This plasmid also contains a 250 bp region of the neomycin phosphotransferase (neo) gene inserted in inverse orientation in the 3' transcribed but untranslated region. Finally, to generate the WEXP2 expression vector, a 2 kb *Sfi* I fragment was amplified from pWRES4 using Sf-1 (SEQ ID No:23) and Sf-2 (SEQ ID No:24) oligonucleotides. This amplified fragment was digested with *Sfi* I and inserted into *Sfi* I linearized pWRES4, generating pWEXP2. This destroys the *Wnt-1* translation initiation codon, and replaces it by a polylinker containing *Nru* I, *Eco* RV, *Sac* II, and *Bst* BI restriction sites, which are unique in pWEXP2.

The WEXP2 - *lacZ* construct was obtained by inserting an end-filled *Bgl* II - *Xho* I *lacZ* fragment isolated from the pSDKlacZpA vector in the *Nru* I cut pWEXP2 expression vector. Similarly, the WEXP2 - *CShh* construct was obtained by inserting an end-filled *Xba*I cDNA fragment containing the full Chick *Shh* coding sequence (SEQ ID No:1) into the *Nru* I cut WEXP2 expression vector.

Oligonucleotide sequences are as follows:

lac1: 5'-AGCTGTCGACGCGGCCGCTACGTAGGTTACCGACGTCAAGCTTAGATCTC-3'
 lac2: 5'-AGCTGAGATCTAAGCTTGACGTCGGTAACCTACGTAGCGGCCGCGTCGAC-3'
 Sf-1: 5'-GATCGGCCAGGCAGGCCTCGCGATATCGTCACCGCGGTATTCGAA-3'
 Sf-2: 5'-AGTGCCAGTCGGGGCCCCCAGGGCCGCGCC-3'

Production And Genotyping Of Transgenic Embryos

Transgenic mouse embryos were generated by microinjection of linear DNA fragments into the male pronucleus of B6CBAF1/J (C57BL/6J X CBA/J) zygotes. CD-1 or B6CBAF1/J females were used as recipients for injected embryos. G₀ mice embryos were collected at 9.5, 10.5, and 11.5 dpc, photographed using an Olympus SZH stereophotomicroscope on Kodak EPY-64T color slide film, then processed as described earlier.

WEXP2-*lacZ* and WEXP2-*CShh* transgenic embryos were identified by PCR analysis of proteinase-K digests of yolk sacs. Briefly, yolk sacs were carefully dissected free from maternal and embryonic tissues, avoiding cross-contamination between littermates, then washed once in PBS. After overnight incubation at 55°C in 50 µl of PCR proteinase-K digestion buffer (McMahon, A.P. et al., (1990) *Cell* 62: 1073-1085). 1 µl of heat-inactivated digest was subjected to polymerase chain reaction (PCR) in a 20 µl volume for 40 cycles as follows: 94°C for 30 seconds, 55°C for 30 seconds, 72°C for 1 minute, with the reaction ingredients described previously (McMahon, A.P. et al., (1990) *Cell* 62: 1073-1085)). In the case of the WEXP2 - *lacZ* transgenic embryos, oligonucleotides 137 (SEQ ID No:25) and

138 (SEQ ID No:26) amplify a 352 bp *lacZ* specific product. In the case of the WEXP2-*CShh* embryos, oligonucleotides WPR2 (*Wnt-1*-specific) (SEQ ID No:27) and 924 (Chick *Shh*-specific) (SEQ ID No:28) amplify a 345 bp fragment spanning the insertion junction of the Chick-*Shh* cDNA in the WEXP2 expression vector. Table 2 summarizes the results of WEXP2-C-*Shh* transgenic studies.

Oligonucleotide sequences are as follows:

137: 5'-TACCACAGCGGATGGTTCGG-3'

138: 5'-GTGGTGGTTATGCCGATCGC-3'

WPR2: 5'-TAAGAGGCCTATAAGAGGCGG-3'

924: 5'-AAGTCAGCCCAGAGGAGACT-3'

(ii) Mouse *hh* Genes

The combined screening of mouse genomic and 8.5 day post coitum (dpc) cDNA libraries identified three mammalian *hh* counterparts (Figure 5A) which herein will be referred to as *Desert*, *Indian* and *Sonic hedgehog* (*Dhh*, *Ihh* and *Shh*, respectively). Sequences encoding *Dhh* (SEQ ID No:2) were determined from analysis of clones identified by low stringency screening of a mouse genomic library. DNA sequencing of one of five overlapping *lambda phage* clones identified three homologous regions encoding a single open reading frame interrupted by introns in identical position to those of the Dros-HH gene (Figure 5A). Splicing across the exon 1/2 boundary was confirmed by polymerase chain reaction (PCR) amplification of first strand cDNA generated from adult testicular RNA. The partial sequence of *Ihh* (SEQ ID No:3) and the complete sequence of *Shh* (SEQ ID No:4) coding regions were determined from the analysis of overlapping cDNA clones isolated from 8.5 dpc cDNA libraries. The longest *Shh* clone, 2.6kb, appears to be full length when compared with the *Shh* transcript present in embryonic RNAs. The 1.8kb partial length *Ihh* cDNA is complete at the 3' end, as evidenced by the presence of a polyadenylation consensus sequence and short poly A tail.

Alignment of the predicted Dros-HH protein sequence (SEQ ID No:34) with those of the mouse *Dhh* (SEQ ID No:9), *Ihh* (SEQ ID No:10) and *Shh* (SEQ ID No:11), and chick *Shh* (SEQ ID No:8) and zebrafish *Shh* (SEQ ID No:12), reveals several interesting features of the *hh*-family (Figure 5A). All the vertebrate *hh*-proteins contain an amino terminal hydrophobic region of approximately 20 amino acids immediately downstream of the initiation methionine. Although the properties of these new *hh* proteins have not been investigated, it is likely that this region constitutes a signal peptide and vertebrate *hhs* are secreted proteins. Signal peptide cleavage is predicted to occur (von Heijne, G., (1986) *Nucleic Acids Research* 14: 4683-4690) just before an absolutely conserved six amino acid stretch, CGPGRG (SEQ ID No:29) (corresponding to residues 85-90)(Figure 5A), in all *hh* proteins. This generates

80

processed mouse *Dhh* (SEQ ID No:9) and *Shh* (SEQ ID No:11) proteins of 41 and 44 kd, respectively. Interestingly, Dros-HH (SEQ ID No:34) is predicted to contain a substantial amino terminal extension beyond the hydrophobic domain suggesting that the *Drosophila* protein enters the secretory pathway by a type II secretory mechanism. This would generate
5 a transmembrane tethered protein which would require subsequent cleavage to release a 43 kd secreted form of the protein. *In vitro* analysis of Dros-HH is consistent with this interpretation (Lee, J.J. et al., (1992) *Cell* 71: 33-50). However, there also appears to be transitional initiation at a second methionine (position 51 of SEQ ID No:34) just upstream of the hydrophobic region (Lee, J.J. et al., (1992) *Cell* 71: 33-50), suggesting that Dros-HH, like
10 its vertebrate counterparts, may also be secreted by recognition of a conventional amino terminal signal peptide sequence.

Data base searches for protein sequences related to vertebrate *hh*'s failed to identify any significant homologies, excepting Dros-HH. In addition, searching the "PROSITE" data bank of protein motifs did not reveal any peptide motifs which are conserved in the different
15 *hh* proteins. Thus, the *hhs* represent a novel family of putative cell signaling molecules.

One feature of the amino acid alignment is the high conservation of *hh* sequences. Vertebrate *hhs* share 47 to 51% amino acid identity with Dros-HH throughout the predicted processed polypeptide sequence (Figure 6). *Dhh* has a slightly higher identity than that of *Ihh* and *Shh* suggesting that *Dhh* may be the orthologue of Dros-HH. Conservation is highest
20 in the amino terminal half of the proteins, indeed, from position 85 (immediately after the predicted shared cleavage site) to 249, 62% of the amino acids are completely invariant amongst the *Drosophila* and vertebrate proteins. Comparison of mouse *Dhh*, *Ihh* and *Shh* where their sequences overlap in this more conserved region, indicates that *Ihh* and *Shh* are more closely related (90% amino acid identity; residues 85 to 266) than with the *Dhh*
25 sequence (80% amino acid identity; residues 85 to 266). Thus, *Ihh* and *Shh* presumably resulted from a more recent gene duplication event.

Comparison of cross species identity amongst *Shh* proteins reveals an even more striking sequence conservation. Throughout the entire predicted processed sequence mouse and chick *Shh* share 84% of amino acid residues (Figure 6). However, in the amino terminal
30 half (positions 85 to 266) mouse and chick are 99% and mouse and zebrafish 94% identical in an 180 amino acid stretch. Conservation falls off rapidly after position 266 (Figure 5A). SEQ ID No:40 shows the consensus sequence in the amino terminal half of all vertebrate *Shh* genes (human, mouse, chicken and zebrafish) identified to date. SEQ ID No:41 shows the consensus sequence in the amino terminal half of vertebrate *hedgehog* genes (*Shh*, *Ihh*, and
35 *Dhh*) identified to date in different species (mouse, chicken, human and zebrafish).

In summary, *hh* family members are likely secreted proteins consisting of a highly conserved amino terminal and more divergent carboxyl terminal halves. The extreme interspecies conservation of the vertebrate *Shh* protein points to likely conservation of *Shh* function across vertebrate species.

5 (iii) *Expression of Mouse Shh at the Axial Midline*

Expression of *Shh* in the mouse was examined in order to explore the role of mouse *Shh* (SEQ ID No:11) in vertebrate development. Northern blots of embryonic and adult RNA samples were probed with a radiolabelled mouse *Shh* cDNA probe. An *Shh* transcript of approximately 2.6kb was detected in 9.5dpc whole embryo RNA, and 9.5 and 10.5dpc brain
10 RNA fractions. No expression was detected in total RNA samples from later embryonic stages. Of the late fetal and adult tissue RNAs examined *Shh* expression was only detected in 16.5dpc and adult lung.

To better define the precise temporal and spatial expression of *Shh* an extensive series of whole mount and serial section *in situ* hybridizations were performed using digoxigenin
15 and ³⁵S-radiolabelled RNA probes, respectively, to mouse embryo samples from 7.25dpc (mid streak egg cylinder stage of gastrulation) to 13.5dpc. No *Shh* expression is detected at mid-gastrulation stages (7.25dpc) prior to the appearance of the node, the mouse counterpart of the amphibian organizer and chick Hensen's node. When the primitive streak is fully extended and the midline mesoderm of the head process is emerging from the node (7.5 to
20 7.75dpc), *Shh* is expressed exclusively in the head process. At late head fold stages, *Shh* is expressed in the node and midline mesoderm of the head process extending anteriorly under the presumptive brain. Just prior to somite formation, *Shh* extends to the anterior limit of the midline mesoderm, underlying the presumptive midbrain. As somites are formed, the embryonic axis extends caudally. The notochord, which represents the caudal extension of
25 the head process, also expresses *Shh*, and expression is maintained in the node.

Interestingly, by 8 somites (8.5dpc) strong *Shh* expression appears in the CNS. Expression is initiated at the ventral midline of the midbrain, above the rostral limit of the head process. By 10 somites CNS expression in the midline extends rostrally in the forebrain and caudally into the hindbrain and rostral spinal cord. Expression is restricted in the
30 hindbrain to the presumptive floorplate, whereas midbrain expression extends ventro-laterally. In the forebrain, there is no morphological floor plate, however ventral *Shh* expression here is continuous with the midbrain. By 15 somites ventral CNS expression is continuous from the rostral limit of the diencephalon to the presumptive spinal cord in somitic regions. Over the next 18 to 24 hrs, to the 25-29 somite stage, CNS expression
35 intensifies and forebrain expression extends rostral to the optic stalks. In contrast to all other CNS regions, in the rostral half of the diencephalon, *Shh* is not expressed at the ventral

midline but in two strips immediately lateral to this area which merge again in the floor of the forebrain at its rostral limit. Expression of *Shh* in both the notochord and floorplate is retained until at least 13.5dpc.

Several groups have recently reported the cloning and expression of vertebrate members of a family of transcription factors, related to the *Drosophila forkhead* gene. One of these, *HNF-3 β* shows several similarities in expression to *Shh* (Sasaki, H. et al., (1993) *Development* 118: 47-59) suggesting that *HNF-3 β* may be a potential regulator of *Shh*. To investigate this possibility, direct comparison of *HNF-3 β* and *Shh* expression was undertaken. *HNF-3 β* transcripts are first detected in the node (as previously reported by Sasaki, H. et al., (1993) *supra*), prior to the emergence of the head process and before *Shh* is expressed. From the node, expression proceeds anteriorly in the head process, similar to *Shh* expression. Activation of *HNF-3 β* within the CNS is first observed at 2-3 somites, in the presumptive mid and hindbrain, prior to the onset of *Shh* expression. By 5 somites, expression in the midbrain broadens ventro-laterally, extends anteriorly into the forebrain and caudally in the presumptive floor plate down much of the neuraxis in the somitic region. Strong expression is maintained at this time in the node and notochord. However, by 10 somites expression in the head process is lost and by 25-29 somites notochordal expression is only present in the most extreme caudal notochord. In contrast to the transient expression of *HNF-3 β* in the midline mesoderm, expression in the floor plate is stably retained until at least 11.5dpc. Thus, there are several spatial similarities between the expression of *HNF-3 β* and *Shh* in both the midline mesoderm and ventral CNS and it is likely that both genes are expressed in the same cells. However, in both regions, *HNF-3 β* expression precedes that of *Shh*. The main differences are in the transient expression of *HNF-3 β* in the head process and notochord and *Shh* expression in the forebrain. Whereas *HNF-3 β* and *Shh* share a similar broad ventral and ventral lateral midbrain and caudal diencephalic expression, only *Shh* extends more rostrally into the forebrain. In general, these results are consistent with a model in which initial activation of *Shh* expression may be regulated by *HNF-3 β* .

The similarity in *Shh* and *HNF-3 β* expression domains is also apparent in the definitive endoderm which also lies at the midline. Broad *HNF-3 β* expression in the foregut pocket is apparent at 5 somites as previously reported by Sasaki, H. et al., (1993) *supra*. *Shh* is also expressed in the endoderm, immediately beneath the forebrain. Both genes are active in the rostral and caudal endoderm from 8 to 11 somites. Whereas *HNF-3 β* is uniformly expressed, *Shh* expression is initially restricted to two ventro-lateral strips of cells. Ventral restricted expression of *Shh* is retained in the most caudal region of the presumptive gut until at least 9.5dpc whereas *HNF-3 β* is uniformly expressed along the dorso-ventral axis. Both genes are expressed in the pharyngeal ectoderm at 9.5dpc and expression is maintained in the gut until at least 11.5dpc. Moreover, expression of *Shh* in the embryonic and adult lung RNA

suggests that endodermal expression of *Shh* may continue in, at least some endoderm derived organs.

(iv) *Expression Of Shh In The Limb*

Expression of *Shh* is not confined to midline structures. By 30-35 somites (9.75dpc),
5 expression is detected in a small group of posterior cells in the forelimb bud. The forelimb buds form as mesenchymal outpocketings on the flanks, opposite somites 8 to 12, at approximately the 17 to 20 somite stage. *Shh* expression is not detectable in the forelimbs until about 30-35 somites, over 12 hours after the initial appearance of the limbs. Expression is exclusively posterior and restricted to mesenchymal cells. By 10.5dpc, both the fore and
10 hindlimbs have elongated substantially from the body flank. At this time *Shh* is strongly expressed in the posterior, distal aspect of both limbs in close association with the overlying ectoderm. Analysis of sections at this stage detects *Shh* expression in an approximately six cell wide strip of posterior mesenchymal cells. In the forelimb, *Shh* expression ceases by 11.5dpc. However, posterior, distal expression is still detected in the hindlimb. No limb
15 expression is detected beyond 12.5dpc.

(v) *Ectopic Expression Of Shh*

Grafting studies carried out principally in the chick demonstrate that cell signals derived from the notochord and floor plate pattern the ventral aspect of the CNS (as described above). In the limb, a transient signal produced by a group of posterior cells in both limb
20 buds, the zone of polarizing activity (ZPA), is thought to regulate patterning across the anterior-posterior axis. Thus, the sequence of *Shh*, which predicts a secreted protein and the expression profile in midline mesoderm, the floor plate and in the limb, suggest that *Shh* signaling may mediate pattern regulation in the ventral CNS and limb.

To determine whether *Shh* may regulate ventral development in the early mammalian
25 CNS, a *Wnt-1* enhancer was used to alter its normal domain of expression. *Wnt-1* shows a dynamic pattern of expression which is initiated in the presumptive midbrain just prior to somite formation. As the neural folds elevate and fuse to enclose the neural tube, *Wnt-1* expression in the midbrain becomes restricted to a tight circle, just anterior of the midbrain, the ventral midbrain and the dorsal midline of the diencephalon, midbrain, myelencephalon and spinal cord (Wilkinson, D.G. et al., (1987) *Cell* 50: 79-88; McMahon, A.P. et al., (1992) *Cell* 69: 581-595; Parr, B.A. et al., (1993) *Development* 119: 247-261).
30

It was determined that essentially normal expression of *lacZ* reporter constructs within the *Wnt-1* expression domain is dependent upon a 5.5kb enhancer region which lies downstream of the *Wnt-1* polyadenylation sequence. A construct was generated for ectopic
35 expression of cDNA clones in the *Wnt-1* domain and tested in transgenics using a *lacZ*

reporter (pWEXP-lacZ; Figure 9). Two of the four G₀ transgenic embryos showed readily detectable β -galactosidase activity, and in both expression occurred throughout the normal *Wnt-1* expression domain. More extensive studies with a similar construct also containing the 5.5kb enhancer gave similar frequencies. Some ectopic expression was seen in newly emerging neural crest cells, probably as a result of perdurance of β -galactosidase RNA or protein in the dorsally derived crest. Thus, the *Wnt-1* expression construct allows the efficient ectopic expression of cDNA sequences in the midbrain and in the dorsal aspect of much of the CNS.

An *Shh* ectopic expression construct (pWEXP-C*Shh*) containing two tandem head to tail copies of a chick *Shh* cDNA was generated (Figure 7). By utilizing this approach, ectopic expression of the chick *Shh* is distinguishable from that of the endogenous mouse *Shh* gene. Chick *Shh* shows a high degree of sequence identity and similar expression to the mouse gene. Thus, it is highly likely that *Shh* function is widely conserved amongst vertebrates, a conclusion further supported by studies of the same gene in zebrafish.

Table 2 shows the results of several transgenic experiments in which the G₀ population was collected at 9.5 to 11.5dpc. Approximately half of the transgenic embryos identified at each stage of development had a clear, consistent CNS phenotype. As we expect, on the basis of control studies using the 5.5kb *Wnt-1* enhancer, that only half the transgenics will express the transgene, it is clear that in most embryos ectopically expressing chick *Shh*, an abnormal phenotype results.

TABLE 2
Summary of WEXP2-Chick *Shh* transgenic studies

Age (dpc)	Number of Embryos	Number of Transgenics	Number of Embryos with CNS phenotype ^a
9.5	37	11	6 (54.5%)
10.5	59	16	8 (50%)
11.5	33	7	3 (42.9%)

Figures in parentheses, refer to the percentage of transgenic embryos with a CNS phenotype

^a In addition one 9.5pc and two 10.5pc transgenic embryos showed non-specific growth retardation, as occurs at low frequency in transgenic studies. These embryos were excluded from further analysis.

At 9.5dpc, embryos with a weaker phenotype show an open neural plate from the mid diencephalon to the myelencephalon. In embryos with a stronger phenotype at the same stage, the entire diencephalon is open and telencephalic and optic development is morphologically abnormal. As the most anterior diencephalic expression of *Wnt-1* is lower than that in more caudal regions, the differences in severity may relate to differences in the level of chick *Shh* expression in different G₀ embryos. At the lateral margins of the open

neural folds, where *Wnt-1* is normally expressed, there is a thickening of the neural tissue extending from the diencephalon to myelencephalon. The cranial phenotype is similar at 10.5 and 11.5 dpc. However, there appears to be a retardation in cranial expansion of the CNS at later stages.

5 In addition to the dorsal cranial phenotype, there is a progressive dorsal phenotype in the spinal cord. At 9.5 dpc, the spinal cord appears morphologically normal, except at extreme rostral levels. However by 10.5dpc, there is a dorsal dysmorphology extending to the fore or hindlimbs. By 11.5 dpc, all transgenic embryos showed a dorsal phenotype along almost the entire spinal cord. Superficially, the spinal cord had a rippled, undulating appearance suggestive of a change in cell properties dorsally. This dorsal phenotype, and the
10 cranial phenotype were examined by histological analysis of transgenic embryos.

Sections through a 9.5dpc embryo with an extreme CNS phenotype show a widespread dorsal perturbation in cranial CNS development. The neural/ectodermal junction in the diencephalon is abnormal. Neural tissue, which has a columnar epithelial morphology
15 quite distinct from the squamous epithelium of the surface ectoderm, appears to spread dorsolaterally. The myelencephalon, like the diencephalon and midbrain, is open rostrally. Interestingly, there are discontinuous dorso-lateral regions in the myelencephalon with a morphology distinct from the normal roof plate regions close to the normal site of *Wnt-1* expression. These cells form a tight, polarized epithelium with basally located nuclei, a
20 morphology similar to the floor plate and distinct from other CNS regions. Differentiation of dorsally derived neural crest occurs in transgenic embryos as can be seen from the presence of cranial ganglia. In the rostral spinal cord, the neural tube appeared distended dorso-laterally which may account for the superficial dysmorphology.

By 11.5dpc, CNS development is highly abnormal along the entire dorsal spinal cord
25 to the hindlimb level. The dorsal half of the spinal cord is enlarged and distended. Dorsal sensory innervation occurs, however, the neuronal trajectories are highly disorganized. Most obviously, the morphology of dorsal cells in the spinal cord, which normally are elongated cells with distinct lightly staining nuclei and cytoplasm, is dramatically altered. Most of the dorsal half of the spinal cord consists of small tightly packed cells with darkly staining nuclei
30 and little cytoplasm. Moreover, there appears to be many more of these densely packed cells, leading to abnormal outgrowth of the dorsal CNS. In contrast, ventral development is normal, as are dorsal root ganglia, whose origins lie in neural cells derived from the dorsal spinal cord.

(vi) *Ectopic Shh Expression Activates Floor Plate Gene Expression*

35 To determine whether ectopic expression of chick *Shh* results in inappropriate activation of a ventral midline development in the dorsal CNS, expression of two floor plate

expressed genes, HNF-3 β and mouse *Shh*, were examined. Whole mounts of 9.5dpc transgenic embryos show ectopic expression of HNF-3 β throughout the cranial *Wnt-1* expression domain. In addition to normal expression at the ventral midline, HNF-3 β transcripts are expressed at high levels, in a circle just rostral to the mid/hindbrain junction, along the dorsal (actually lateral in unfused brain folds) aspects of the midbrain and, more weakly, in the roof plate of the myelencephalon. No expression is observed in the metencephalon which does not express *Wnt-1*. Thus, ectopic expression of *Shh* leads to the activation of HNF-3 β throughout the cranial *Wnt-1* expression domain.

The relationship between chick *Shh* expression and the expression of HNF-3 β in serial sections was also examined. Activation of HNF-3 β in the brain at 9.5 and 10.5dpc is localized to the dorsal aspect in good agreement with the observed ectopic expression of chick *Shh*. Interestingly mouse *Shh* is also activated dorsally. Thus, two early floor plate markers are induced in response to chick *Shh*.

From 9.5dpc to 11.5dpc, the spinal cord phenotype becomes more severe. The possibility that activation of a floor plate pathway may play a role in the observed phenotype was investigated. In contrast to the brain, where ectopic HNF-3 β and *Shh* transcripts are still present, little or no induction of these floor plate markers is observed. Thus, although the dorsal spinal cord shows a widespread transformation in cellular phenotype, this does not appear to result from the induction of floor plate development.

Example 3

Chick Sonic Hedgehog Mediates ZPA Activity

(i) Experimental Procedures

Retinoic Acid Bead Implants

Fertilized white Leghorn chicken eggs were incubated to stage 20 and then implanted with AG1-X2 ion exchange beads (Biorad) soaked in 1 mg/ml retinoic acid (RA, Sigma) as described by Tickle, C. et al., (1985) *Dev. Biol* 109: 82-95. Briefly, the beads were soaked for 15 min in 1mg/ml RA in DMSO, washed twice and implanted under the AER on the anterior margin of the limb bud. After 24 or 36 hours, some of the implanted embryos were harvested and fixed overnight in 4% paraformaldehyde in PBS and then processed for whole mount in situ analysis as previously described. The remainder of the animals were allowed to develop to embryonic day 10 to confirm that the dose of RA used was capable of inducing mirror image duplications. Control animals were implanted with DMSO soaked beads and showed no abnormal phenotype or gene expression.

Plasmids

87

Unless otherwise noted, all standard cloning techniques were performed according to Ausubel, F.M. et al., (1989) *Current Protocols in Molecular Biology* (N.Y.: Greene Publishing Assoc. and Wiley Interscience), and all enzymes were obtained from Boehringer Mannheim Biochemicals. pHH-2 is a cDNA contain the entire coding region of chicken *Sonic hedgehog* (SEQ ID No:1). RCASBP(A) and RCASBP(E) are replication-competent retroviral vectors which encode viruses with differing host ranges. RCANBP(A) is a variant of RCASBP(A) from which the second splice acceptor has been removed. This results in a virus which can not express the inserted gene and acts as a control for the effects of viral infection (Hughes, S.H. et al., (1987) *J. Virol.* 61: 3004-3012; Fekete, D. et al., (1993) *Mol. Cell. Biol.* 13: 2604-2613). RCASBP/AP(E) is version of RCASBP(E) containing a human placental alkaline phosphatase cDNA (Fekete, D. et al., (1993b) *Proc. Natl. Acad. Sci. USA* 90: 2350-2354). SLAX13 is a pBluescript SK+ derived plasmid with a second Cla I restriction site and the 5' untranslated region of v-src (from the adaptor plasmid CLA12-Nco, Hughes, S.H. et al., (1987) *J. Virol.* 61: 3004-3012) cloned 5' of the EcoRI (and ClaI) site in the pBluescript polylinker. RCASBP plasmids encoding *Sonic* from either the first (M1) or second (M2) methionine (at position 4) were constructed by first shuttling the 1.7kb *Sonic* fragment of pHH-2 into SLAX-13 using oligonucleotides to modify the 5' end of the cDNA such that either the first or second methionine is in frame with the NcoI site of SLAX-13. The amino acid sequence of *Sonic* is not mutated in these constructs. The M1 and M2 *Sonic* ClaI fragments (v-src 5'UTR:*Sonic*) were each then subcloned into RCASBP(A), RCANBP(A) and RCASBP(E), generating Sonic/RCAS-A1, Sonic/RCAS-A2, Sonic/RCAN-A1, Sonic/RCAN-A2, Sonic/RCAS-E1 and Sonic/RCAS-E2.

Chick Embryos, Cell Lines And Virus Production

All experimental manipulations were performed on standard specific-pathogen free White Leghorn chick embryos (S-SPF) from closed flocks provided fertilized by SPAFAS (Norwich, Conn). Eggs were incubated at 37.5°C and staged according to Hamburger, V. et al., (1951) *J. Exp. Morph.* 88: 49-92. All chick embryo fibroblasts (CEF) were provided by C. Cepko. S-SPF embryos and CEFs have previously been shown to be susceptible to RCASBP(A) infection but resistant to RCASBP(E) infection (Fekete, D. et al., (1993b) *Proc. Natl. Acad. Sci. USA* 90: 2350-2354). Line 15b CEFs are susceptible to infection by both RCASBP(A) and (E). These viral host ranges were confirmed in control experiments. CEF cultures were grown and transfected with retroviral vector DNA as described (Morgan, B.A. et al., (1993) *Nature* 358: 236-239; Fekete, D. et al., (1993b) *Proc. Natl. Acad. Sci. USA* 90: 2350-2354). All viruses were harvested and concentrated as previously described (Morgan, B.A. et al., (1993) *Nature* 358: 236-239; Fekete, D. et al., (1993b) *Proc. Natl. Acad. Sci. USA* 90: 2350-2354) and had titers of approximately 10^8 cfu/ml.

Cell Implants

A single 60mm dish containing line 15b CEFs which had been infected with either RCASBP/AP(E), Sonic/RCAS-E1 or Sonic/RCAS-E2 were grown to 50-90% confluence, lightly trypsinized and then spun at 1000 rpm for 5 min in a clinical centrifuge. The pellet was resuspended in 1 ml media, transferred to a microcentrifuge tube and then microcentrifuged for 2 min at 2000 rpm. Following a 30 min incubation at 37° C, the pellet was respun for 2 min at 2000 rpm and then lightly stained in media containing 0.01% Nile blue sulfate. Pellet fragments of approximately 300µm x 100µm x 50µm were implanted as a wedge to the anterior region of *hh* stage 19-23 wing buds (as described by Riley, B.B. et al., (1993) *Development* 118: 95-104). At embryonic day 10, the embryos were harvested, fixed in 4% paraformaldehyde in PBS, stained with Alcian green, and cleared in methyl salicylate (Tickle, C. et al., (1985) *Dev. Biol* 109: 82-95).

Viral Infections

Concentrated Sonic/RCAS-A2 or Sonic/RCAN-A2 was injected under the AER on the anterior margin of stage 20-22 wing buds. At 24 or 36 hours post-infection, the embryos were harvested, fixed in 4% paraformaldehyde in PBS and processed for whole mount *in situ* analysis as previously described.

(ii) Co-Localization Of Sonic Expression And Zpa Activity

ZPA activity has been carefully mapped both spatially and temporally within the limb bud (Honig, L.S. et al., (1985) *J. Embryol. exp. Morph.* 87: 163-174). In these experiments small blocks of limb bud tissue from various locations and stages of chick embryogenesis (Hamburger, V et al., (1951) *J. Exp. Morph.* 88: 49-92) were grafted to the anterior of host limb buds and the strength of ZPA activity was quantified according to degrees of digit duplication. Activity is first weakly detected along the flank prior to limb bud outgrowth. The activity first reaches a maximal strength at stage 19 in the proximal posterior margin of the limb bud. By stage 23 the activity extends the full length of the posterior border of the limb bud. The activity then shifts distally along the posterior margin so that by stage 25 it is no longer detectable at the base of the flank. The activity then fades distally until it is last detected at stage 29.

This detailed map of endogenous polarizing activity provided the opportunity to determine the extent of the correlation between the spatial pattern of ZPA activity and *Sonic* expression over a range of developmental stages. Whole mount *in situ* hybridization was used to assay the spatial and temporal pattern of *Sonic* expression in the limb bud. *Sonic* expression is not detected until stage 17, at the initiation of limb bud formation, at which time it is weakly observed in a punctate pattern reflecting a patchy expression in a few cells.

From that point onwards the *Sonic* expression pattern exactly matches the location of the ZPA, as determined by Honig, L.S. et al., (1985) *J. Embryol. exp. Morph.* 87: 163-174, both in position and in intensity of expression.

(iii) *Induction Of Sonic Expression By Retinoic Acid*

5 A source of retinoic acid placed at the anterior margin of the limb bud will induce ectopic tissue capable causing mirror-image duplications (Summerbell, D. et al., (1983) *In Limb Development and Regeneration* (N.Y.: Ala R. Liss) pp. 109-118; Wanek, N. et al., (1991) *Nature* 350: 81-83). The induction of this activity is not an immediate response to retinoic acid but rather takes approximately 18 hours to develop (Wanek, N. et al., (1991) 10 *Nature* 350: 81-83). When it does develop, the polarizing activity is not found surrounding the implanted retinoic acid source, but rather is found distal to it in the mesenchyme along the margin of the limb bud (Wanek, N. et al., (1991) *Nature* 350: 81-83).

 If *Sonic* expression is truly indicative of ZPA tissue, then it should be induced in the ZPA tissue which is ectopically induced by retinoic acid. To test this, retinoic acid-soaked 15 beads were implanted in the anterior of limb buds and the expression of *Sonic* after various lengths of time using whole-mount *in situ* hybridization was assayed. As the limb bud grows, the bead remains imbedded proximally in tissue which begins to differentiate. Ectopic *Sonic* expression is first detected in the mesenchyme 24 hours after bead implantation. This expression is found a short distance from the distal edge of the bead. By 20 36 hours *Sonic* is strongly expressed distal to the bead in a stripe just under the anterior ectoderm in a mirror-image pattern relative to the endogenous *Sonic* expression in the posterior of the limb bud.

(iv) *Effects Of Ectopic Expression Of Sonic On Limb Patterning*

 The normal expression pattern of *Sonic*, as well as that induced by retinoic acid, is 25 consistent with *Sonic* being a signal produced by the ZPA. To determine whether *Sonic* expression is sufficient for ZPA activity, the gene was ectopically expressed within the limb bud. In most of the experiments we have utilized a variant of a replication-competent retroviral vector called RCAS (Hughes, S.H. et al., (1987) *J. Virol.* 61: 3004-3012)) both as a vehicle to introduce the *Sonic* sequences into chick cells and to drive their expression. The 30 fact that there exists subtypes of avian retroviruses which have host ranges restricted to particular strains of chickens was taken advantage of to control the region infected with the *Sonic*/RCAS virus (Weiss, R. (et al.) (1984) *RNA Tumor Viruses*, Vol. 1 Weiss et al. eds., (N.Y.: Cold Spring Harbor Laboratories) pp. 209-260); Fekete, D. et al., (1993a) *Mol. Cell. Biol.* 13: 2604-2613). Thus a vector with a type E envelope protein (RCAS-E, Fekete, D. et al., (1993b) *Proc. Natl. Acad. Sci. USA* 90: 2350-2354) is unable to infect the cells of the 35 SPAFAS outbred chick embryos routinely used in our lab. However, RCAS-E is able to

infect cells from chick embryos of line 15b. In the majority of experiments, primary chick embryo fibroblasts (CEFs) prepared from line 15b embryos *in vitro* were infected. The infected cells were pelleted and implanted into a slit made in the anterior of S-SPF host limb buds. Due to the restricted host range of the vector, the infection was thus restricted to the graft and did not spread through the host limb bud.

To determine the fate of cells implanted and to control for any effect of the implant procedure, a control RCAS-E vector expressing human placental alkaline phosphatase was used. Alkaline phosphatase expression can be easily monitored histochemically and the location of infected cells can thus be conveniently followed at any stage. Within 24 hours following implantation the cells are dispersed proximally and distally within the anterior margin of the limb bud. Subsequently, cells are seen to disperse throughout the anterior portion of the limb and into the flank of the embryo.

Limb buds grafted with alkaline phosphatase expressing cells or uninfected cells give rise to limbs with structures indistinguishable from unoperated wild type limbs. Such limbs have the characteristic anterior-to-posterior digit pattern 2-3-4. ZPA grafts give rise to a variety of patterns of digits depending on the placement of the graft within the bud (Tickle, C. et al., (1975) *Nature* 254: 199-202) and the amount of tissue engrafted (Tickle, C. (1981) *Nature* 289: 295-298). In some instances the result can be as weak as the duplication of a single digit 2. However, in optimal cases the ZPA graft evokes the production of a full mirror image duplication of digits 4-3-2-2-3-4 or 4-3-2-3-4 (see Figure 8). A scoring system has been devised which rates the effectiveness of polarizing activity on the basis of the most posterior digit duplicated: any graft which leads to the development of a duplication of digit 4 has been defined as reflecting 100% polarizing activity (Honig, L.S. et al., (1985) *J. Embryol. Exp. Morph.* 87: 163-174).

Grafts of 15b fibroblasts expressing *Sonic* resulted in a range of ZPA-like phenotypes. In some instances the resultant limbs deviate from the wild type solely by the presence of a mirror-image duplication of digit 2. The most common digit phenotype resulting from grafting *Sonic*-infected CEF cells is a mirror-image duplication of digits 4 and 3 with digit 2 missing: 4-3-3-4. In many such cases the two central digits appear fused in a 4-3/3-4 pattern. In a number of the cases the grafts induced full mirror-image duplications of the digits equivalent to optimal ZPA grafts 4-3-2-2-3-4. Besides the digit duplications, the ectopic expression of *Sonic* also gave rise to occasional duplications of proximal elements including the radius or ulna, the humerus and the coracoid. While these proximal phenotypes are not features of ZPA grafts, they are consistent with an anterior-to-posterior respecification of cell fate. In some instances, most commonly when the radius or ulna was duplicated, more complex digit patterns were observed. Typically, an additional digit 3 was formed distal to a duplicated radius.

The mirror-image duplications caused by ZPA grafts are not limited to skeletal elements. For example, feather buds are normally present only along the posterior edge of the limb. Limbs exhibiting mirror-image duplications as a result of ectopic *Sonic* expression have feather buds on both their anterior and posterior edges, similar to those observed in ZPA grafts.

While ZPA grafts have a powerful ability to alter limb pattern when placed at the anterior margin of a limb bud, they have no effect when placed at the posterior margin (Saunders, J.W. et al., (1968) *Epithelial-Mesenchymal Interaction*, Fleischmayer and Billingham, eds. (Baltimore: Williams and Wilkins) pp. 78-97). Presumably, the lack of posterior effect is a result of polarizing activity already being present in that region of the bud. Consistent with this, grafts of *Sonic* expressing cells placed in the posterior of limb buds never result in changes in the number of digits. Some such grafts did produce distortions in the shape of limb elements, the most common being a slight posterior curvature in the distal tips of digits 3 and 4 when compared to wild type wings.

(v) *Effect Of Ectopic Sonic Expression On Hoxd Gene Activity*

The correct expression of *Hoxd* genes is part of the process by which specific skeletal elements are determined (Morgan, B.A. et al., (1993) *Nature* 358: 236-239). A transplant of a ZPA into the anterior of a chick limb bud ectopically activates sequential transcription of *Hoxd* genes in a pattern which mirrors the normal sequence of *Hoxd* gene expression (Nohno, T. et al., (1991) *Cell* 64: 1197-1205; Izpisua-Belmonte, J.C. et al., (1991) *Nature* 350: 585-589). Since ectopic *Sonic* expression leads to the same pattern duplications as a ZPA graft, we reasoned that *Sonic* would also lead to sequential activation of *Hoxd* genes.

To test this hypothesis, anterior buds were injected with Sonic/RCAS-A2, a virus which is capable of directly infecting the host strains of chicken embryos. This approach does not strictly limit the region expressing *Sonic* (being only moderately controlled by the timing, location and titer of viral injection), and thus might be expected to give a more variable result. However, experiments testing the kinetics of viral spread in infected limb buds indicate that infected cells remain localized near the anterior margin of the bud for at least 48 hours. *Hoxd* gene expression was monitored at various times post infection by whole mount in situ hybridization. As expected, these genes are activated in a mirror-image pattern relative their expression in the posterior of control limbs. For example, after 36 hours *Hoxd-13* is expressed in a mirror-image symmetrical pattern in the broadened distal region of infected limb buds. Similar results were obtained with other *Hoxd* genes (manuscript in preparation).

Example 4*A Functionally Conserved Homolog of Drosophila Hedgehog is Expressed in Tissues With Polarizing Activity in Zebrafish Embryos**(i) Experimental Procedures*5 *Cloning and Sequencing*

Approximately 1.5×10^6 plaques of a 33h zebrafish embryonic λ gt11 cDNA library were screened by plaque hybridization at low stringency (McGinnis, W. et al., (1984) *Nature* 308: 428-433) using a mix of two *hh* sequences as a probe: a *Drosophila hh* 400bp EcoRI fragment and a murine *Ihh* 264bp BamHI-EcoRI exon 2 fragment. Four clones were isolated and subcloned into the EcoRI sites of pUC18 T3T7 (Pharmacia). Both strands of clone 8.3 were sequenced using nested deletions (Pharmacia) and internal oligonucleotide primers. DNA sequences and derived amino acid sequences were analyzed using "Geneworks" (Intelligenetics) and the GCG software packages.

PCR amplification

15 Degenerate oligonucleotides *hh*5.1 (SEQ ID No:30) and *hh*3.3 (SEQ ID No:31) were used to amplify genomic zebrafish DNA

hh 5.1: AG(CA)GITG(CT)AA(AG)GA(AG)(CA)(AG)I(GCT)IAA

hh 3.3: CTCIACIGCIA(GA)ICK(GT)IGCIA

PCR was performed with an initial denaturation at 94°C followed by 35 cycles of 47°C for 1 min, 72°C for 2min and 94°C for 1 min with a final extension at 72°C. Products were subcloned in pUC18 (Pharmacia).

In Situ Hybridization

In situ hybridizations of zebrafish embryos were performed as described in Oxtoby, E. et al., (1993) *Nuc. Acids REs.* 21: 1087-1095 with the following modifications: Embryos were rehydrated in ethanol rather than methanol series; the proteinase K digestion was reduced to 5 min and subsequent washes were done in PBTw without glycine; the antibody was preadsorbed in PBTw, 2mg/ml BSA without sheep serum; and antibody incubation was performed in PBTw, 2mg/ml BSA. *Drosophila* embryos were processed and hybridized as previously described.

30 *Histology*

Stained embryos were dehydrated through ethanol:butanol series, as previously described (Godsave, S.F. et al., (1988) *Development* 102: 555-566), and embedded in Fibrowax. 8 μ m sections were cut on an Anglian rotary microtome

RNA Probe Synthesis

For analysis of *Shh* expression, two different templates were used with consistent results; (i) *phh[c]* 8.3 linearized with Bgl II to transcribe an antisense RNA probe that excludes the conserved region, and (ii) *phh[c]* 8.3 linearized with Hind III to transcribe an antisense RNA that covers the complete cDNA. All *in situ* hybridizations were performed with the latter probe which gives better signal. Other probes were as follows: *Axial* DraI-linearized p6TIN (Strähle, U. et al., (1993) *Genes & Dev.* 7: 1436-1446) using T3 RNA polymerase. *gsc* linearized with EcoR1 and transcribed with T7; *pax 2* Bam HI-linearized pcF16 (Krauss, S. et al., (1991) *Development* 113: 1193-1206) using T7 RNA polymerase. *In situ* hybridizations were performed using labelled RNA at a concentration of 1 ng/ml final concentration. Antisense RNA probes were transcribed according to the manufacturer's protocol (DIG RNA Labelling Kit, BCL).

Zebrafish Strains

Wild type fish were bred from a founder population obtained from the Goldfish Bowl, Oxford. The mutant *cyclops* strain bl6 and the mutant *notail* strains bl60 and bl95 were obtained from Eugene, Oregon. Fish were reared at 28°C on a 14h light/10h dark cycle.

RNA Injections

The open reading frame of *Shh* was amplified by PCR, using oligonucleotides 5'-CTGCAGGGATCCACCATGCGGCTTTTGACGAG-3' (SEQ ID No:32), which contains a consensus Kozak sequence for translation initiation, and 5'-CTGCAGGGATC-CTTATTCCACACGAGGGATT-3' (SEQ ID No:33), and subcloned into the BglII site of pSP64T (Kreig, P.A. et al., (1984) *Nuc.Acids Res.* 12: 7057-7070). This vector includes 5' and 3' untranslated *Xenopus* β -Globin sequences for RNA stabilization and is commonly used for RNA injections experiments in *Xenopus*. *In vitro* transcribed *Shh* RNA at a concentration of approximately 100 μ g/ml was injected into a single cell of naturally spawned zebrafish embryos at one-cell to 4-cell stages using a pressure-pulsed Narishige microinjector. The injected volume was within the picolitre range. Embryos were fixed 20 to 27 hrs after injection in BT-Fix (Westerfield, M. (1989) *The Zebrafish Book*, (Eugene: The University of Oregon Press)) and processed as described above for whole-mount *in situ* hybridizations with the *axial* probe.

Transgenic Drosophila

An EcoR1 fragment, containing the entire *Shh* ORF, was purified from the plasmid *phh[c]*8.3 and ligated with phosphatased EcoR1 digested transformation vector pCaSpeRhs (Thummel, C.S. et al., (1988) *Gene* 74: 445-456). The recombinant plasmid, pHS *Shh* containing the *Shh* ORF in the correct orientation relative to the heat shock promoter, was

selected following restriction enzyme analysis of miniprep DNA from transformed colonies and used to transform *Drosophila* embryos using standard microinjection procedures (Roberts, D.B. (1986), *Drosophila, A Practical Approach*, Roberts, D.B., ed., (Oxford: IRL Press) pp. 1-38).

5 *Ectopic Expression In Drosophila Embryos*

Embryos carrying the appropriate transgenes were collected over 2 hr intervals, transferred to thin layers of 1% agarose on glass microscope slides and incubated in a plastic Petri dish floating in a water bath at 37°C for 30 min intervals. Following heat treatment, embryos were returned to 25°C prior to being fixed for *in situ* hybridization with DIG
10 labelled single stranded *Shh*, *wg* or *ptc* RNA probes as previously described (Ingham et al., (1991) *Curr. Opin. Genet. Dev.* 1: 261-267).

(ii) *Molecular Cloning Of Zebrafish Hedgehog Homologues*

In an initial attempt to isolate sequences homologous to *Drosophila hh*, a zebrafish genomic DNA library was screened at reduced stringency with a partial cDNA, *hhPCR4.1*,
15 corresponding to the first and second exons of the *Drosophila* gene (Mohler, J. et al., (1992) *Development* 115: 957-971). This screen proved unsuccessful; however, a similar screen of a mouse genomic library yielded a single clone with significant homology to *hh*., subsequently designated *Ihh*. A 264bp BamHI-EcoRI fragment from this lambda clone containing sequences homologous to the second exon of the *Drosophila* gene was subcloned and,
20 together with the *Drosophila* partial cDNA fragment, used to screen a λ gt11 zebrafish cDNA library that was prepared from RNA extracted from 33h old embryos. This screen yielded four clones with overlapping inserts the longest of which is 1.6kb in length, herein referred to as *Shh* (SEQ ID No:5).

25 (iii) *A Family Of Zebrafish Genes Homologous To The Drosophila Segment Polarity Gene, Hedgehog*

Alignment of the predicted amino acid sequences of *Shh* (SEQ ID No:12) and *hh* (SEQ ID No:34) revealed an identity of 47%, confirming that *Shh* is a homolog of the *Drosophila* gene. A striking conservation occurs within exon 2: an 80 amino acid long domain shows 72% identity between *Shh* and Dros-HH. (Figure 9A). This domain is also
30 highly conserved in all *hh*-related genes cloned so far and is therefore likely to be essential to the function of *hh* proteins. A second domain of approximately 30 amino acids close to the carboxy-terminal end, though it shows only 61% amino-acid identity, possesses 83% similarity between *Shh* and *hh* when allowing for conservative substitutions and could also, therefore, be of functional importance (Figure 9B). Although putative sites of post-
35 translational modification can be noted, their position is not conserved between *Shh* and *hh*.

Lee, J.J. et al., (1992) *Cell* 71: 33-50, identified a hydrophobic stretch of 21 amino acids flanked downstream by a putative site of signal sequence cleavage (predicted by the algorithm of von Heijne, G. (1986) *Nuc. Acids Res.* 11) close to the amino-terminal end of *hh*. Both the hydrophobic stretch and the putative signal sequence cleavage sites of *hh*, which suggest it to be a signaling molecule, are conserved in *Shh*. In contrast to *hh*, *Shh* does not extend N-terminally to the hydrophobic stretch.

Using degenerate oligonucleotides corresponding to amino-acids flanking the domain of high homology between Dros-HH and mouse *Ihh* exons 2 described above, fragments of the expected size were amplified from zebrafish genomic DNA by PCR. After subcloning and sequencing, it appeared that three different sequences were amplified, all of which show high homology to one another and to Dros-HH (Figure 10). One of these corresponds to *Shh* therein referred to as 2-hh(a) (SEQ ID No:16) and 2hh(b) (SEQ ID No:17), while the other two represent additional zebrafish *hh* homologs (SEQ ID No:5). cDNAs corresponding to one of these additional homologs have recently been isolated, confirming that it is transcribed. Therefore, *Shh* represents a member of a new vertebrate gene family.

(iv) *Shh* Expression In The Developing Zebrafish Embryo

Gastrula stages

Shh expression is first detected at around the 60% epiboly stage of embryogenesis in the dorsal mesoderm. Transcript is present in a triangular shaped area, corresponding to the embryonic shield, the equivalent of the amphibian organizer, and is restricted to the inner cell layer, the hypoblast. During gastrulation, presumptive mesodermal cells involute to form the hypoblast, and converge towards the future axis of the embryo, reaching the animal pole at approximately 70% epiboly. At this stage, *Shh* -expressing cells extend over the posterior third of the axis, and the signal intensity is not entirely homogeneous, appearing stronger at the base than at the apex of the elongating triangle of cells.

This early spatial distribution of *Shh* transcript is reminiscent of that previously described for *axial*, a *forkhead*-related gene; however, at 80% epiboly, *axial* expression extends further towards the animal pole of the embryo and we do not see *Shh* expression in the head area at these early developmental stages.

By 100% epiboly, at 9.5 hours of development, the posterior tip of the *Shh* expression domain now constitutes a continuous band of cells that extends into the head. To determine the precise anterior boundary of *Shh* expression, embryos were simultaneously hybridized with probes of *Shh* and *pax-2* (previously *pax[b]*), the early expression domain of which marks the posterior midbrain (Krauss, S. et al. (1991) *Development* 113: 1193-1206). By this stage, the anterior boundary of the *Shh* expression domain is positioned in the centre of the

animal pole and coincides approximately with that of *axial*. At the same stage, prechordal plate cells expressing the homeobox gene *gooseoid* (*gsc*) overlap and underlay the presumptive forebrain (Statchel, S.E. et al., (1993) *Development* 117: 1261-1274). Whereas *axial* is also thought to be expressed in head mesodermal tissue at this stage, we cannot be certain whether *Shh* is expressed in the same cells. Sections of stained embryos suggest that in the head *Shh* may by this stage be expressed exclusively in neuroectodermal tissue.

(v) *Somitogenesis*

By the onset of somitogenesis (approximately 10.5h of development), *Shh* expression in the head is clearly restricted to the ventral floor of the brain, extending from the tip of the diencephalon caudally through the hindbrain. At this stage, expression of *axial* has also disappeared from the head mesoderm and is similarly restricted to the floor of the brain; in contrast to *Shh*, however, it extends only as far as the anterior boundary of the midbrain. At this point, *gsc* expression has become very weak and is restricted to a ring of cells that appear to be migrating away from the dorsal midline.

As somitogenesis continues, *Shh* expression extends in a rostral-caudal progression throughout the ventral region of the central nervous system (CNS). Along the spinal cord, the expression domain is restricted to a single row of cells, the floor plate, but gradually broadens in the hindbrain and midbrain to become 5-7 cells in diameter, with a triangular shaped lateral extension in the ventral diencephalon and two strongly staining bulges at the tip of the forebrain, presumably in a region fated to become hypothalamus.

As induction of *Shh* in the floor plate occurs, expression in the underlying mesoderm begins to fade away, in a similar manner to *axial* (Strähle, U. et al., (1993) *Genes & Dev.* 7: 1436-1446). This downregulation also proceeds in a rostral to caudal sequence, coinciding with the changes in cell shape that accompany notochord differentiation. By the 22 somite stage, mesodermal *Shh* expression is restricted to the caudal region of the notochord and in the expanding tail bud where a bulge of undifferentiated cells continue to express *Shh* at relatively high levels. Expression in the midbrain broadens to a rhombic shaped area; cellular rearrangements that lead to the 90° kink of forebrain structures, position hypothalamic tissue underneath the ventral midbrain. These posterior hypothalamic tissues do not express *Shh*. In addition to *Shh* expression in the ventral midbrain, a narrow stripe of expressing cells extends dorsally on either side of the third ventricle from the rostral end of the *Shh* domain in the ventral midbrain to the anterior end of, but not including, the epiphysis. The most rostral *Shh* expressing cells are confined to the hypothalamus. In the telencephalon, additional *Shh* expression is initiated in two 1-2 cell wide stripes.

By 36 hours of development, *Shh* expression in the ventral CNS has undergone further changes. While expression persists in the floor plate of the tailbud, more rostrally

located floor plate cells in the spinal cord cease to express the gene. In contrast, in the hindbrain and forebrain *Shh* expression persists and is further modified.

At 26-28h, *Shh* expression appears in the pectoral fin primordia, that are visible as placode like broadenings of cells underneath the epithelial cell layer that covers the yolk. By 33 hrs of development high levels of transcript are present in the posterior margin of the pectoral buds; at the same time, expression is initiated in a narrow stripe at the posterior of the first gill. Expression continues in the pectoral fin buds in lateral cells in the early larva. At this stage, *Shh* transcripts are also detectable in cells adjacent to the lumen of the foregut.

(vi) *Expression Of Shh In Cyclops And Notail Mutants*

Two mutations affecting the differentiation of the *Axial* tissues that express *Shh* have been described in zebrafish embryos homozygous for the *cyclops* (*cyc*) mutation lack a differentiated floorplate (Hatta, K. et al., (1991) *Nature* 350: 339-341). By contrast, homozygous *notail* (*ntl*) embryos are characterized by a failure in notochord maturation and a disruption of normal development of tail structures (Halpern, M.E. et al., (1993) *Cell* 75: 99-111).

A change in *Shh* expression is apparent in *cyc* embryos as early as the end of gastrulation; at this stage, the anterior limit of expression coincides precisely with the two *pax-2* stripes in the posterior midbrain. Thus, in contrast to wild-type embryos, no *Shh* expression is detected in midline structures of the midbrain and forebrain. By the 5 somite stage, *Shh* transcripts are present in the notochord which at this stage extends until rhombomere 4; however, no expression is detected in more anterior structures. Furthermore, no *Shh* expression is detected in the ventral neural keel, in particular in the ventral portions of the midbrain and forebrain.

At 24 hours of development, the morphologically visible *cyc* phenotype consists of a fusion of the eyes at the midline due to the complete absence of the ventral diencephalon. As at earlier developmental stages, *Shh* expression is absent from neural tissue. *Shh* expression in the extending tail bud of wild-type embryos is seen as a single row of floor plate cells throughout the spinal cord. In a *cyc* mutant, no such *Shh* induction occurs in cells of the ventral spinal cord with the exception of some scattered cells that show transient expression near the tail. Similarly, no *Shh* expression is seen rostrally in the ventral neural tube. However, a small group of *Shh* expressing cells is detected underneath the epiphysis which presumably correspond to the dorsal-most group of *Shh* expressing cells in the diencephalon of wild-type embryos.

In homozygous *notail* (*ntl*) embryos, no *Shh* staining is seen in mesodermal tissue at 24 hours of development, consistent with the lack of a notochord in these embryos; by

contrast, expression throughout the ventral CNS is unaffected. At the tail bud stage, however, just prior to the onset of somitogenesis, *Shh* expression is clearly detectable in notochord precursor cells.

5 (vii) *Injection Of Synthetic Shh Transcripts Into Zebrafish Embryos Induces Expression Of A Floor Plate Marker*

To investigate the activity of *Shh* in the developing embryo, an over-expression strategy, similar to that employed in the analysis of gene function in *Xenopus*, was adopted. Newly fertilized zebrafish eggs were injected with synthetic *Shh* RNA and were fixed 14 or 24 hours later. As an assay for possible changes in cell fate consequent upon the ectopic activity of *Shh*, we decided to analyze *Axial* expression, since this gene serves as a marker for cells in which *Shh* is normally expressed. A dramatic, though highly localized ectopic expression of *Axial* in a significant proportion (21/80) of the injected embryos fixed after 24 hours of development is observed. Affected embryos show a broadening of the *Axial* expression domain in the diencephalon and ectopic *Axial* expression in the midbrain; however, in no case has ectopic expression in the telencephalon or spinal cord been observed. Many of the injected embryos also showed disturbed forebrain structures, in particular smaller ventricles and poorly developed eyes. Amongst embryos fixed after 14h, a similar proportion (8/42) exhibit the same broadening and dorsal extension of the *Axial* stripe in the diencephalon as well as a dorsal extension of *Axial* staining in the midbrain; again, no changes in *Axial* expression were observed caudal to the hindbrain with the exception of an increased number of expressing cells at the tip of the tail.

(viii) *Overexpression Of Shh In Drosophila Embryos Activates The hh-Dependent Pathway*

In order to discover whether the high degree of structural homology between the *Drosophila* and zebrafish *hh* genes also extends to the functional level, an overexpression system was used to test the activity of *Shh* in flies. Expression of Dros-HH driven by the HSP70 promoter results in the ectopic activation of both the normal targets of *hh* activity; the *wg* transcriptional domain expands to fill between one third to one half of each parasegment whereas *ptc* is ectopically activated in all cells except those expressing *en* (Ingham, P.W. (1993) *Nature* 366:560-562). To compare the activities of the fly and fish genes, flies transgenic for a HS *Shh* construct were generated described above and subjected to the same heat shock regime as H *Shh* transgenic flies. HS *Shh* embryos fixed immediately after the second of two 30 min heat shocks exhibit ubiquitous transcription of the *Shh* cDNA. Similarly treated embryos were fixed 30 or 90 min after the second heat shock and assayed for *wg* or *ptc* transcription. Both genes were found to be ectopically activated in a similar manner to that seen in heat shocked H *Shh* embryos; thus, the zebrafish *Shh* gene can activate the same pathway as the endogenous *hh* gene.

Example 5*Cloning, Expression and Localization of Human Hedgehogs**(i) Experimental Procedures*5 *Isolation of human hedgehog cDNA clones.*

Degenerate nucleotides used to clone chick *Shh* (Riddle *et al.*, (1993) *Cell* 75:1401-1416) were used to amplify by nested PCR human genomic DNA. The nucleotide sequence of these oligos is as follows:

10 vHH5O:5'-GGAATTCCCAG(CA)GITG(CT)AA(AG)GA(AG)(CA)(AG)I(GCT)TIAA-3'
(SEQ ID NO:18);
vHH3O:5'-TCATCGATGGACCCA(GA)TC(GA)AAICCGC(TC)TC-3' (SEQ ID NO:19);
vHH3I:5'-GCTCTAGAGCTCIACIGCIA(GA)IC(GT)IGGIA-3' (SEQ ID NO:20)

15 The expected 220 bp PCR product was subcloned into pGEM7zf (Promega) and sequenced using Sequenase v2.0 (U.S. Biochemicals). One clone showed high nucleotide similarity to mouse *Ihh* and mouse *Shh* sequence (Echelard *et al.*, (1993) *Cell* 75:1417-1430) and it was used for screening a human fetal lung 5'-stretch plus cDNA library (Clontech) in λ gt10 phage. The library was screened following the protocol suggested by the company and
20 two positive plaques were identified, purified, subcloned into pBluescript SK+ (Stratagene) and sequenced, identifying them as the human homologues of *Shh* (SEQ ID NO:6) and *Ihh* (SEQ ID NO:7).

One clone contained the full coding sequence of a human homolog of *Shh* as well as 150 bp of 5' and 36 bp of 3' untranslated sequence. The other clone, which is the human
25 homolog of *Ihh*, extends from 330 bp 3' of the coding sequence to a point close to the predicted boundary between the first and second exon. The identity of these clones was determined by comparison to the murine and chick genes. The protein encoded by human *Shh* has 92.4% overall identity to the mouse *Shh*, including 99% identity in the amino-terminal half. The carboxyl-terminal half is also highly conserved, although it contains short
30 stretches of 16 and 11 amino acids not present in the mouse *Shh*. The human *Ihh* protein is 96.8% identical to the mouse *Ihh*. The two predicted human proteins are also highly related, particularly in their amino-terminal halves where they are 91.4% identical. They diverge significantly in their carboxyl halves, where they show only 45.1% identity. The high level of similarity in the amino portion of all of these proteins implies that this region encodes
35 domains essential to the activity of this class of signaling molecules.

Northern blotting

Multiple Tissue Northern Blot (Clontech) prepared from poly A+RNA isolated from human adult tissues was hybridized with either full length ³²P-labeled human *Shh* clone or
5 ³²P-labeled human *Ihh* clone following the protocol suggested by the company.

Digoxigenin in situ hybridization.

Sections: tissues from normal human second trimester gestation abortus specimens were washed in PBS and fixed overnight at 4°C paraformaldehyde in PBS, equilibrated 24
10 hours at 4°C in 50% sucrose in PBS and then placed in 50% sucrose in oct for one hour before embedding in oct. Cryostat sections (10-25 mm) were collected on superfrost plus slides (Fisher) and frozen at -80°C until used. Following a postfixation in 4% paraformaldehyde the slides were processed as in Riddle et al., (1993) *Cell* 75:1401-1416 with the following alterations: proteinase K digestion was performed at room temperature
15 from 1-15 minutes (depending on section thickness), prehybridization, hybridization and washes time was decreased to 1/10 of time.

Whole-mounts: tissues from normal second trimester human abortus specimens were washed in PBS, fixed overnight at 4°C in 4% paraformaldehyde in PBS and then processed as in Riddle et al., (1993) *Cell* 75:1401-1416.

20

Isolation of an Shh P1 clone.

The human *Shh* gene was isolated on a P1 clone from a P1 library (Pierce and Sternberg, 1992) by PCR (polymerase chain reaction) screening. Two oligonucleotide primers were derived from the human *Shh* sequence. The two oligonucleotide primers used for
25 PCR were:

SHHF5'-ACCGAGGGCTGGGACGAAGATGGC-3' (SEQ ID NO:43)

SHR5'-CGCTCGGTCGTACGGCATGAACGAC-3' (SEQ ID NO:44)

The PCR reaction was carried using standard conditions as described previously (Thierfelder et al., 1994) except that the annealing temperature was 65°C. These primers amplified a 119
30 bp fragment from human and P1 clone DNA. The P1 clone was designated SHHP1. After the P1 clone was isolated these oligonucleotides were used as sequencing primers. A 2.5Kb *EcoRI* fragment that encoded a CA repeat was subcloned from this P1 clone using methods described previously (Thierfelder et al. 1994). Oligonucleotide primers that amplified this CA repeat sequence were fashioned from the flanking sequences:

35 SHHCAF5'-ATGGGGATGTGTGTGGTCAAGTGTA-3' (SEQ ID NO:45)

SHHCAR5'-TTCACAGACTCTCAAAGTGTATTTT-3' (SEQ ID NO:46)

Mapping the human Ihh and Shh genes.

The human *Ihh* gene was mapped to chromosome 2 using somatic cell hybrids from NIGMS mapping pannel 2 (GM10826B).

5 The *Shh* gene was mapped to chromosome 7 using somatic cell hybrids from NIGMS mapping panel 2 (GM10791 and GM10868).

Linkage between the limb deformity locus on chromosome 7 and the *Shh* gene was demonstrated using standard procedures. Family LD has been described previously (Tkukurov *et al.*, (1994) *Nature Genet.* 6:282-286). A CA repeat bearing sequence near the
10 *Shh* gene was amplified from the DNA of all members of Family LD by PCR using the SHHCAF and SHHCAR primers. Linkage between the CA repeat and the LD disease gene segregating in Family LD was estimated by the MLINK program (Oct, 1967). Penetrance was set at 100% and the allele frequencies were determined using unrelated spouses in the LD family.

15

Interspecific Backcross Mapping.

Interspecific backcross progeny were generated by mating (C57BL/6J x *M. spretus*) F1 females and C57BL/6J males as described (Copeland and Jenkins, (1991) *Trends Genet.* 7:113-118). A total of 205 N2 mice were used to map the *Ihh* and *Dhh* loci. DNA isolation,
20 restriction enzyme digestions, agarose gel electrophoresis, Southern blot transfer and hybridization were performed essentially as described (Jenkins *et al.*, (1982) *J. Virol.* 43:26-36). All blots were prepared with Hybond-N+ nylon membrane (Amersham). The probe, an ~ 1.8kb *EcoRI* fragment of mouse cDNA, detected a major fragment of 8.5 kb in C57BL/6j (B) DNA and a major fragment 6.0 kb in *M. spretus* (S) DNA following digestion with *BglII*.
25 The *Shh* probe, an ~ 900 bp *SmaI* fragment of mouse cDNA, detected *HincII* fragments of 7.5 and 2.1 kb (B) as well as 4.6 and 2.1 (S). The *Dhh* probe, and ~ 800 bp *BamHI/EcoRI* fragment of mouse genomic DNA, detected major fragments of 4.7 and 1.3 kb (B) and 8.2 and 1.3 kb (S) following digestion with *SphI*. The presence or absence of *M. spretus* specific fragments was followed in backcross mice.

30

A description of the probes and RFLPs for loci used to position the *Ihh*, *Shh* and *Dhh* loci in the interspecific backcross has been reported. These include: *Fnl*, *Vil* and *Acrg*, chromosome 1 (Wilkie *et al.*, (1993) *Genomics* 18:175-184), *Gnail*, *En2*, *Il6*, chromosomes 5 (Miao *et al.*, (1994) *PNAS USA* 91:11050-11054) and *Pdgfb*, *Gdc1* and *Rarg*, chromosome 15 (Brannan *et al.*, (1992) *Genomics* 13:1075-1081). Recombination distances were
35 calculated as described (Green, (1981) Linkage, recombination and mapping. In "*Genetics and Probability in Animal Breeding Experiments*", pp. 77-113, Oxford University Press, NY) using the computer program SPRETUS MADNESS. Gene order was determined by

minimizing the number of recombination events required to explain the allele distribution patterns.

(ii) *Expression of Human Shh and Ihh*

To investigate the tissue distribution of *Shh* and *Ihh* expression, poly(A)+RNA samples from various adult human tissues were probed with the two cDNA clones. Of the tissues tested, an *Ihh*-specific message of ~2.7 kb is only detected in liver and kidney. *Shh* transcripts was not detected in the RNA from any of the adult tissues tested. All the samples contained approximately equal amounts of intact RNA, as determined by hybridization with a control probe.

The *hedgehog* family of genes were identified as mediators of embryonic patterning in flies and vertebrates. No adult expression of these genes had previously been reported. These results indicate that *Ihh* additionally plays a role in adult liver and kidney. Since the *hedgehog* genes encode intercellular signals, *Ihh* may function in coordinating the properties of different cell types in these organs. *Shh* may also be used as a signaling molecule in the adult, either in tissues not looked at here, or at levels too low to be detected under these conditions.

In situ hybridization was used to investigate the expression of *Shh* in various mid-gestational human fetal organs. *Shh* expression is present predominantly in endoderm derived tissues: the respiratory epithelium, collecting ducts of the kidney, transitional epithelium of the ureter, hepatocytes, and small intestine epithelium. *Shh* was not detectable in fetal heart or placental tissues. The intensity of expression is increased in primitive differentiating tissues (renal blastema, base villi, branching lung buds) and decreased or absent in differentiated tissues (e.g. glomeruli). *Shh* expression is present in the mesenchyme immediately abutting the budding respiratory tubes. The non-uniform pattern of *Shh* expression in hepatocytes is consistent with expression of other genes in adult liver (Dingemanse *et al.*, (1994) *Differentiation* 56:153-162). The base of villi, the renal blastema, and the lung buds are all regions expressing *Shh* and they are areas of active growth and differentiation, suggesting *Shh* is important in these processes.

(iii) *The Chromosomal Map Location of Human Shh and Ihh.*

Since *Shh* is known to mediate patterning during the development of the mouse and chick and the expression of *Shh* and *Ihh* are suggestive of a similar role in humans, mutations in these genes would be expected to lead to embryonic lethality or congenital defects. One way of investigating this possibility is to see whether they are genetically linked to any known inherited disorders.

Shh- and *Ihh*-specific primers were designed from their respective sequences and were used in PCR reactions on a panel of rodent-human somatic cell hybrids. Control rodent DNA did not amplify specific bands using these primers. In contrast, DNA from several rodent-human hybrids resulted in PCR products of the appropriate size allowing us to assign *Shh* to chromosome 7q and *Ihh* to chromosome 2.

One of the central roles of chick *Shh* is in regulating the anterior-posterior axis of the limb. A human congenital polysyndactyly has recently been mapped to chromosome 7q36 (Tsukurov *et al.*, (1994) *Nature Genet.* 6:282-286; Heutink *et al.*, (1994) *Nature Genet.* 6:287-291). The phenotype of this disease is consistent with defects that might be expected from aberrant expression of *Shh* in the limb. Therefore, the chromosomal location of *Shh* was mapped more precisely, in particular in relation to the polysyndactyly locus.

A P1 phage library was screened using the *Shh* specific primers for PCR amplification and clone SHHP1 was isolated. Clone SHHP1 contained *Shh* sequence. A Southern blot of an *EcoRI* digest of this phage using [CA]/[GT] probe demonstrated that a 2.5 Kb *EcoRI* fragment contained a CA repeat. Nucleotide sequence analysis of this subcloned *EcoRI* fragment demonstrated that the CA repeat lay near the *EcoRI* sites. Primers flanking the CA repeat were designed and used to map the location of *Shh* relative to other markers on 7q in individuals of a large kindred with complex polysyndactyly (Tsukurov *et al.*, (1994) *Nature Genet.* 6:282-286). *Shh* maps close to D75550 on 7q36, with no recombination events seen in this study. It is also extremely close to, but distinct from, the polysyndactyly locus with one recombination event observed between them (maximum lod score = 4.82, Θ = 0.05). One unaffected individual (pedigree ID V-10 in Tsukurov *et al.*, (1994) *Nature Genet.* 6:282-286) has the *Shh* linked CA repeat allele found in all affected family members. No recombination was observed between the locus *En2* and the *Shh* gene (maximum lod score = 1.82, Θ = 0.0).

(iv) *Chromosomal mapping of the Murine Ihh, Shh and Dhh genes.*

The murine chromosomal location of *Ihh*, *Shh* and *Dhh* was determined using an interspecific backcross mapping panel derived from crosses of [(C57BL/6J x *M. spretus*)F1 X C57BL/6J] mice. cDNA fragments from each locus were used as probes in Southern blot hybridization analysis of C57BL/6J and *M. spretus* genomic DNA that was separately digested with several different restriction enzymes to identify informative restriction fragment length polymorphisms (RFLPs) useful for gene mapping. The strain distribution pattern of each RFLP in the interspecific backcross was then determined by following the presence or absence of RFLPs specific for *M. spretus* in backcross mice.

Ihh mapped to the central region of mouse chromosome 1, 2.7 cM distal of *Fnl* and did not recombine with *Vil* in 190 animals typed in common, suggesting that the two loci are within 1.6 cM (upper 95% confidence level) (Fig. 16). *Shh* mapped to the proximal region of

mouse chromosome 5, 0.6 cM distal of *En2* and 1.9 cM proximal of *I16* in accordance to Chang *et al.*, (1994) *Development* 120:3339-3353. *Dhh* mapped to the very distal region of mouse chromosome 15, 0.6 cM distal of *Gdc1* and did not recombine with *Rarg* in 160 animals typed in common, suggesting that the two loci are within 1.9 cM of each other (upper 5 95% confidence level) (Fig. 16).

Interspecific maps of chromosome 1, 5 and 15 were compared with composite mouse linkage maps that report the map location of many uncloned mouse mutations (compiled by M.T. Davisson, T.H. Roderick, A.L. Hillyard and D.P. Doolittle and provided from GBASE, a computerized database maintained at The Jackson Laboratory, Bar Harbor, ME). The 10 *hemimelic extra-toe (Hx)* mouse mutant maps 1.1 cM distal to *En2* on chromosome 5 (Martin *et al.*, (1990) *Genomics* 6:302-308), a location in close proximity to where *Shh* has been positioned. *Hx* is a dominant mutation which results in preaxial polydactyly and hemimelia affecting all four limbs (Dickie, (1968) *Mouse News Lett* 38:24; Knudsen and Kochhar, (1981) *J. Embryol. Exp. Morph.* 65: Suppl. 289-307). *Shh* has previously been shown to be 15 expressed in the limb (Echelard *et al.*, (1993) *Cell* 75:1417-1430). To determine whether *Shh* and *Hx* are tightly linked we followed their distribution in a backcross panel in which *Hx* was segregating. Two recombinants between *Shh* and *Hx* were identified, thus excluding the possibility that the two loci are allelic and these observations are again consistent with those of Chang *et al.*, (1994) *Development* 120:3339-3353. While there are several other mutations 20 in the vicinity of *Ihh* and *Dhh*, none is an obvious candidate for an alteration in the corresponding gene.

The central region of mouse chromosome 1 shares homology with human chromosome 2q (summarized in Fig. 16). Placement of *Ihh* in this interval suggests the human homolog of *Ihh* will reside on 2q, as well. Similarly, it is likely that human homolog 25 of *Dhh* will reside on human chromosome 12q.

Example 6

Proteolytic Processing Yields Two Secreted Forms of Sonic Hedgehog

(i) Experimental Procedures

In vitro Translation and Processing

Mouse and chick *sonic hedgehog* coding sequences were inserted into the vector pSP64T (kindly provided by D. Melton) which contains an SP6 phage promoter and both 5' and 3' untranslated sequences derived from the *Xenopus laevis* β -Globin gene. After restriction endonuclease digestion with *Sal I* to generate linear templates, RNA was 35 transcribed *in vitro* using SP6 RNA polymerase (Promega, Inc.) in the presence of 1 mM cap structure analog (m⁷G(5')ppp(5')Gm; Boehringer-Mannheim, Inc.) Following digestion with

105

RQ1 DNase I (Promega, Inc.) to remove the DNA template, transcripts were purified by phenol:chloroform extraction and ethanol precipitation.

Rabbit reticulocyte lysate (Promega, Inc.) was used according to the manufacturer's instructions. For each reaction, 12.5 µl of lysate was programmed with 0.5-2.0 µg of *in vitro* transcribed RNA. The reactions contained 20 µCi of Express labeling mix (NEN/DuPont, Inc.) were included. To address processing and secretion *in vitro*, 1.0-2.0 µl of canine pancreatic microsomal membranes (Promega, Inc.) were included in the reactions. The final reaction volume of 25 µl was incubated for one hour at 30°C. Aliquots of each reaction (between 0.25 and 3.0 µl) were boiled for 3 minutes in Laemmli sample buffer (LSB: 125 mM Tris-HCl [pH 6.8]; 2% SDS; 1% 2-mercaptoethanol; 0.25 mg/ml bromophenol blue) before separating on a 15% polyacrylamide gel. Fixed gels were processed for fluorography using EnHance (NEN/DuPont, Inc.) as described by the manufacturer.

Glycosylation was addressed by incubation with Endoglycosidase H (Endo H; New England Biolabs, Inc.) according to the manufacturer's directions. Reactions were carried out for 1-2 hr at 37°C before analyzing reaction products by polyacrylamide gel electrophoresis (PAGE).

Xenopus Oocyte Injection and Labeling

Oocytes were enzymatically defolliculated and rinsed with OR2 (50 mM HEPES [pH 7.2], 82 mM NaCl, 2.5 mM KCl, 1.5 mM Na₂HPO₄). Healthy stage six oocytes were injected with 30 ng of *in vitro* transcribed, capped mouse *Shh* RNA (prepared as described above). Following a 2 hr recovery period, healthy injected oocytes and uninjected controls were cultured at room temperature in groups of ten in 96-well dishes containing 0.2 ml of OR2 (supplemented with 0.1 mg/ml Gentamicin and 0.4 mg/ml BSA) per well. The incubation medium was supplemented with 50 µCi of Express labeling mix. Three days after injection, the culture media were collected and expression of *Shh* protein analyzed by immunoprecipitation. Oocytes were rinsed several times in OR2 before lysing in TENT (20 mM Tris-HCl [pH 8.0]; 150 mM NaCl, 2mM EDTA; 1% Triton-X-100; 10 µl/oocyte) supplemented with 1 µg/ml aprotinin, 2 µg/ml leupeptin and 1mM phenylmethylsulfonylfluoride (PMSF). After centrifugation at 13000 x g for 10 minutes at 4°C, soluble protein supernatants were recovered and analyzed by immunoprecipitation (see below).

Cos Cell Transfection and Labeling

Cos cells were cultured in Dulbecco's Modified Eagle Medium (DMEM; Sigma, Inc.) supplemented with 10% fetal bovine serum (Gibco/BRL), 2 mM L-Glutamine (Gibco/BRL) and 50 mU/ml penicillin and 50 µg/ml streptomycin (Gibco/BRL). Subconfluent cos cells in

106

35 mm or 60 mm dishes (Falcon, Inc.) were transiently transfected with 2 mg or 6 mg supercoiled plasmid DNA, respectively. Between 42 and 44 hr post-transfection, cells were labeled for 4-6 hr in 0.5 ml (35 mm dishes) or 1.5 ml (60 mm dishes) serum-free DMEM lacking Cysteine and Methionine (Gibco/BRL) and supplemented with 125 μ Ci/ml each of Express labeling mix and L-35S-Cysteine (NEN/DuPont). After labeling, media were collected and used for immunoprecipitation. Cells were rinsed with cold PBS and lysed in the tissue culture dishes by the addition of 0.5 ml (35 mm dishes) or 1.5 ml (60 mm dishes) TENT (with protease inhibitors as described above) and gentle rocking for 30 minutes at 4°C. Lysates were cleared by centrifugation (13000 x g for 5 min. at 4°C) and the supernatants were analyzed by immunoprecipitation (see below).

Baculovirus Production and Infection

A recombinant baculovirus expressing mouse sonic *hedgehog* with a myc epitope tag inserted at the carboxy terminus was generated using the Baculogold kit (Pharmingen, Inc.). The initial virus production used Sf 9 cells, followed by two rounds of amplification in High Five cells (Invitrogen, Inc.) in serum-free medium (ExCell 401; Invitrogen, Inc.). A baculovirus lacking *Shh* coding sequences was also constructed as a control. For protein induction, High Five cells were infected at a multiplicity of approximately 15. Three days later, medium and cells were collected by gentle pipetting. Cells were collected by centrifugation (1000 x g) and the medium was recovered for Western blot analysis. Cell pellets were washed twice in cold PBS and lysed in TENT plus protease inhibitors (see above) by rotating for 30 minutes at 4°C in a microcentrifuge tube. The lysate was cleared as described above prior to Western blotting.

Western Blotting

For Western blotting, 0.25 ml samples of media (1% of the total) were precipitated with TCA and redissolved in 15 μ l of LSB. Cell lysate samples (1% of total) were brought to a final volume of 15 μ l with water and concentrated (5X) LSB. Samples were boiled 5 minutes prior to separation on a 15% acrylamide gel. Proteins were transferred to PVDF membrane (Immobilon-P; Millipore, Inc.) and blocked in BLOTTO (5% w/v non-fat dried milk in PBS) containing 0.2% Tween-20. Hybridoma supernatant recognizing the human c-myc epitope (9E10; Evan, G.I. et al., (1985) *Mol. Cell. Biol.* 5:3610-3616) was added at a dilution of 1:200 for one hour followed by a 1:5000 dilution of Goat anti-Mouse-Alkaline phosphatase conjugate (Promega, Inc.) for 30 minutes. Bands were visualized using the Lumi-Phos 530 reagent (Boehringer-Mannheim) according to the manufacturer's directions.

Immunoprecipitation

Cell lysates (*Xenopus* oocytes or cos cells) were brought to 0.5 ml with TENT (plus protease inhibitors as above). Media samples (OR2 or DMEM) were cleared by centrifugation at 13000 x g for 5 min. (4°C) and 10X TENT was added to a final concentration of 1X (final volume: 0.5-1.5 ml). The c-myc monoclonal antibody hybridoma supernatant was added to 1/20 of the final volume. Samples were rotated for 1 hr at 4°C., then 0.1 ml of 10% (v/v) protein A-Sepharose CL-4B (Pharmacia, Inc.) was added. Samples were rotated an additional 14-16 h. Immune complexes were washed 4 times with 1.0 ml TENT. Immunoprecipitated material was eluted and denatured by boiling for 10 minutes in 25 µl 1X LSB. Following centrifugation, samples were separated on 15% acrylamide gels and processed for fluorography as described previously. Samples for Endo H digestion were eluted and denatured by boiling for 10 minutes in the provided denaturation buffer followed by digestion with Endo H for 1-2 hr at 37°C. Concentrated (SX) LSB was added and the samples were processed for electrophoresis as described.

For immunoprecipitation with the anti-mouse *Shh* serum, samples (Cos cell lysates and DMEM) were precleared by incubating 1 hr on ice with 3 µl pre-immune serum, followed by the addition of 0.1 ml 10% (v/v) Protein A-Sepharose. After rotating for 1 hr at 4°C, supernatants were recovered and incubated for 1 hr on ice with 3µl depleted anti-mouse *Shh* serum (see below). Incubation with Protein A-Sepharose, washing, elution and electrophoresis were then performed as described above.

Immunofluorescent Staining of Cos Cells

Twenty-four hours after transfection, cells were transferred to 8-chamber slides (Lab-Tek, Inc.) and allowed to attach an additional twenty-four hours. Cells were fixed in 2% paraformaldehyde/0.1% glutaraldehyde, washed in PBS and permeabilized in 1% Triton-X-100 (Munro, S. and Pelham, H.R.B., (1987) *Cell* 48:899-907). After washing in PBS, cells were treated for 10 minutes in 1 mg/ml sodium borohydride. Cells were incubated with the c-myc monoclonal antibody hybridoma supernatant (diluted 1:10) and the affinity purified mouse Sonic *hedgehog* antiserum (diluted 1:4) for 45 minutes followed by incubation in 1:100 Goat-anti Mouse IgG-RITC plus 1:100 Goat anti Rabbit IgG FITC (Southern Biotechnology Associates, Inc.) for 45 minutes. DAPI (Sigma, Inc.) was included at 0.3 µg/ml. The slides were mounted in Slo-Fade (Molecular Probes, Inc.) and photographed on a Leitz DMR compound microscope.

Antibody Production and Purification

A PCR fragment encoding amino acids 44-143 of mouse Sonic *hedgehog* was cloned in frame into the *Eco* RI site of pGEX-2T (Pharmacia, Inc.). Transformed bacteria were

induced with IPTG and the fusion protein purified on a Glutathione-Agarose affinity column (Pharmacia, Inc.) according to the manufacturer's instructions. Inoculation of New Zealand White rabbits, as well as test and production bleeding were carried out at Hazelton Research Products, Inc.

5 To deplete the serum of antibodies against Glutathione-S-transferase (GST) and bacterial proteins, a lysate of *E. coli* transformed with pGEX-2T and induced with IPTG was coupled to Affi-Gel 10 (Bio-Rad, Inc.) The serum was incubated in batch for two hours with the depletion matrix before centrifugation (1000 x g for 5 min.) and collection of the supernatant. To make an affinity matrix, purified bacterially expressed protein corresponding
10 to the amino terminal two-thirds of mouse Sonic *hedgehog* was coupled to Affi-Gel 10 (Bio-Rad, Inc.). The depleted antiserum was first adsorbed to this matrix in batch, then transferred to a column. The matrix was washed with TBST (25 mM Tris-HCl [pH 7.5], 140 mM NaCl, 5 mM KCl, 0.1% Triton-X-100), and the purified antibodies were eluted with ten bed volumes of 0.15 M Glycine [pH 2.5]. The solution was neutralized with one volume of 1 M
15 Tris-HCl [pH 8.0], and dialyzed against 160 volumes of PBS.

Other antibodies have been generated against *hedgehog* proteins and three polyclonal rabbit antisera obtained to *hh* proteins can be characterized as follows: Ab77 -reacts only with the carboxyl processed chick *Shh* peptide (27 kd); Ab79 -reacts with amino processed chick, mouse and human *Shh* peptide (19 kd). Weakly reacts with 27 kd peptide from chick and
20 mouse. Also reacts with mouse *Ihh*; and Ab80 -reacts with only amino peptide (19kd) of chick, mouse and human.

(ii) *In Vitro Translated Sonic Hedgehog is Proteolytically Processed and Glycosylated*

The open reading frames of chick and mouse *Shh* encode primary translation products
25 of 425 and 437 amino acids, respectively, with predicted molecular masses of 46.4 kilodaltons (kDa) and 47.8 kDa (Echelard, Y. et al., (1993) *Cell* 75:1417-1430; Riddle, R.D. et al., (1993) *Cell* 75:1401-1416). Further examination of the protein sequences revealed a short stretch of amino terminal residues (26 for chick, 24 for mouse) that are highly hydrophobic and are predicted to encode signal peptides. Removal of these sequences would
30 generate proteins of 43.7 kDa (chick *Shh*) and 45.3 kDa (mouse *Shh*). Also, each protein contains a single consensus site for N-linked glycosylation (Tarentino, A.L. et al., (1989) *Methods Cell Biol.* 32:111-139) at residue 282 (chick) and 279 (mouse). These features of the *Shh* proteins are summarized in Figure 11.

A rabbit reticulocyte lysate programmed with *in vitro* translated messenger RNA
35 encoding either chick or mouse *Shh* synthesizes proteins with molecular masses of 46 kDa and 47 kDa, respectively. These values are in good agreement with those predicted by

examination of the amino acid sequences. To examine posttranslational modifications of *Shh* proteins, a preparation of canine pancreatic microsomal membranes was included in the translation reactions. This preparation allows such processes as signal peptide cleavage and core glycosylation. When the *Shh* proteins are synthesized in the presence of these membranes, two products with apparent molecular masses of approximately 19 and 28 kDa (chick), or 19 and 30 kDa (mouse) are seen in addition to the 46 kDa and 47 kDa forms. When the material synthesized in the presence of the membranes is digested with Endoglycosidase H (Endo H), the mobilities of the two larger proteins are increased. The apparent molecular masses of the Endo H digested forms are 44 kDa and 26 kDa for chick *Shh*, and 45 kDa and 27 kDa for mouse *Shh*. The decrease in the molecular masses of the largest proteins synthesized in the presence of the microsomal membranes after Endo H digestion is consistent with removal of the predicted signal peptides. The mobility shift following Endo H treatment indicates that N-linked glycosylation occurs, and that the 26 kDa (chick) and 27 kDa (mouse) proteins contain the glycosylation sites.

The appearance of the two lower molecular weight bands (hereafter referred to as the "processed forms") upon translation in the presence of microsomal membranes suggests that a proteolytic event in addition to signal peptide cleavage takes place. The combined molecular masses of the processed forms (19 kDa and 26 kDa for chick; 19 kDa and 27 kDa for mouse) add up to approximately the predicted masses of the signal peptide cleaved proteins (44 kDa for chick and 45 kDa for mouse) suggesting that only a single additional cleavage occurs.

The mouse *Shh* protein sequence is 12 amino acid residues longer than the chick sequence (437 versus 425 residues). Alignment of the chick and mouse *Shh* protein sequences reveals that these additional amino acids are near the carboxy terminus of the protein (Echelard, Y. et al., (1993) *Cell* 75:1417-1430). Since the larger of the processed forms differ in molecular mass by approximately 1 kDa between the two species, it appears that these peptides contain the carboxy terminal portions of the *Shh* proteins. The smaller processed forms, whose molecular masses are identical, presumably consist of the amino terminal portions.

(iii) Secretion of *Shh* Peptides

To investigate the synthesis of *Shh* proteins in vivo, the mouse protein was expressed in several different eukaryotic cell types. In order to detect synthesized protein, and to facilitate future purification, the carboxy terminus was engineered to contain a twenty-five amino acid sequence containing a recognition site for the thrombin restriction protease followed by a ten amino acid sequence derived from the human c-myc protein and six consecutive histidine residues. The c-myc sequence serves as an epitope tag allowing

110

detection by a monoclonal antibody (9E10; Evan, G.I. et al., (1985) *Mol. Cell Biol.* 5:3610-3616). The combined molecular mass of the carboxy terminal additions is approximately 3 kDa.

Xenopus laevis oocytes

5 Immunoprecipitation with the c-myc antibody detects several proteins in lysates of metabolically labeled *Xenopus laevis* oocytes injected with *Shh* mRNA. Cell lysates and medium from ³⁵S labeled oocytes injected with RNA encoding mouse *Shh* with the c-myc epitope tag at the at the carboxy terminus, or from control oocytes were analyzed by
10 immunoprecipitation with c-myc monoclonal antibody. A band of approximately 47 kDa is seen, as is a doublet migrating near 30 kDa. Treatment with Endo H increases the mobility of the largest protein, and resolves the doublet into a single species of approximately 30 kDa. These observations parallel the behaviors seen *in vitro*. Allowing for the added mass of the carboxy terminal additions, the largest protein would correspond to the signal peptide cleaved
15 form, while the doublet would represent the glycosylated and unglycosylated larger processed form. Since the epitope tag was placed at the carboxy terminus of the protein, the identity of the 30 kDa peptide as the carboxy terminal portion of *Shh* is confirmed. Failure to detect the 19 kDa species supports its identity as an amino terminal region of the protein.

To test whether *Shh* is secreted by *Xenopus* oocytes, the medium in which the injected oocytes were incubated was probed by immunoprecipitation with the c-myc
20 antibody. A single band migrating slightly more slowly than the glycosylated larger processed form was observed. This protein is insensitive to Endo H. This result is expected since most secreted glycoproteins lose sensitivity to Endo H as they travel through the Golgi apparatus and are modified by a series of glycosidases (Kornfeld, R. and Kornfeld, S., (1985) *Annu. Rev. Biochem.* 54:631-664). The enzymatic maturation of the Asn-linked carbohydrate
25 moiety could also explain the slight decrease in mobility of the secreted larger protein versus the intracellular material. Following Endo H digestion, a band with a slightly lower mobility than the signal peptide cleaved protein is also apparent, suggesting that some *Shh* protein is secreted without undergoing proteolytic processing. Failure to detect this protein in the medium without Endo H digestion suggests heterogeneity in the extent of carbohydrate
30 modification in the Golgi preventing the material from migrating as a distinct band. Resolution of this material into a single band following Endo H digestion suggests that the carbohydrate structure does not mature completely in the Golgi apparatus. Structural differences between the unprocessed protein and the larger processed form could account for this observation (Kornfeld, R. and Kornfeld, S., (1985) *Annu. Rev. Biochem.* 54:631-664).

Cos cells

///

The behavior of mouse *Shh* in a mammalian cell type was investigated using transfected cos cells. Synthesis and secretion of the protein was monitored by immunoprecipitation using the c-myc antibody. Transfected cos cells express the same Sonic *hedgehog* species that were detected in the injected *Xenopus* oocytes, and their behavior following Endo H digestion is also identical. Furthermore, secretion of the 30 kDa glycosylated form is observed in cos cells, as well as the characteristic insensitivity to Endo H after secretion. Most of the secreted protein co-migrates with the intracellular, glycosylated larger processed form, but a small amount of protein with a slightly lower mobility is also detected in the medium. As in the *Xenopus* oocyte cultures, some *Shh* which has not undergone proteolytic processing is evident in the medium, but only after Endo H digestion.

Baculovirus infected cells

To examine the behavior of the mouse *Shh* protein in an invertebrate cell type, and to potentially purify *Shh* peptides, a recombinant baculovirus was constructed which placed the *Shh* coding sequence, with the carboxy terminal tag, under the control of the baculoviral Polyhedrin gene promoter. When insect cells were infected with the recombinant baculovirus, *Shh* peptides could be detected in cell lysates and medium by Western blotting with the c-myc antibody.

The *Shh* products detected in this system were similar to those described above. However, virtually no unprocessed protein was seen in cell lysates, nor was any detected in the medium after Endo H digestion. This suggests that the proteolytic processing event occurs more efficiently in these cells than in either of the other two cell types or the *in vitro* translation system. A doublet corresponding to the glycosylated and unglycosylated 30 kDa forms is detected, as well as the secreted, Endo I resistant peptide as seen in the other expression systems. Unlike the other systems, however, all of the secreted larger processed form appears to comigrate with the glycosylated intracellular material.

(iv) Secretion of a Highly Conserved Amino Terminal Peptide

To determine the behavior of the amino terminal portion of the processed Sonic *hedgehog* protein, the c-myc epitope tag was positioned 32 amino acids after the putative signal peptide cleavage site (Figure 12). Cos cells were transfected with *Shh* expression constructs containing the c-myc tag at the carboxy terminus or near the amino terminus. When this construct was expressed in cos cells, both the full length protein and the smaller processed form (approximately 20 kDa due to addition of the c-myc tag) were detected by immunoprecipitation of extracts from labeled cells. However, the 20 kDa product is barely

detected in the medium. In cells transfected in parallel with the carboxy terminal c-myc tagged construct, the full length and 30 kDa products were both precipitated from cell lysates and medium as described earlier.

As the amino terminal c-myc tag may affect the secretion efficiency of the smaller processed form, the expression of this protein was examined in cos cells using an antiserum directed against amino acids 44 through 143 of mouse *Shh* (Figure 12). After transfection with the carboxy-terminal c-myc tagged construct, immunoprecipitation with the anti-*Shh* serum detected a very low level of the smaller processed form in the medium despite a strong signal in the cell lysate. This recapitulates the results with the myc antibody.

To examine the subcellular localization of *Shh* proteins, cos cells were transfected with the carboxy terminal tagged *Shh* construct and plated on multi-chamber slides, fixed and permeabilized. The cells were incubated simultaneously with the anti-*Shh* serum and the c-myc antibody followed by FITC conjugated Goat anti-Rabbit-IgG and RITC conjugated Goat anti-Mouse-IgG. DAPI was included to stain nuclei. Strong perinuclear staining characteristic of the Golgi apparatus was observed with the anti-*Shh* serum. The same subcellular region was also stained using the c-myc antibody. The coincidence of staining patterns seen with the two antibody preparations suggest that the low level of the smaller processed form detected in the medium is not due to its retention in the endoplasmic reticulum.

(v) *Hedgehog Processing*

In summary, the results discussed above demonstrate that the mouse and chick *Shh* genes encode secreted glycoproteins which undergo additional proteolytic processing. Data indicate that this processing occurs in an apparently similar fashion in a variety of cell types suggesting that it is a general feature of the *Shh* protein, and not unique to any particular expression system. For mouse *Shh*, data indicate that both products of this proteolytic processing are secreted. These observations are summarized in Figure 13.

It was observed that the 19 kDa amino peptide accumulates to a lower level in the medium than the 27 kDa carboxyl peptide. This may reflect inefficient secretion or rapid turnover of this species once secreted. Alternatively, the smaller form may associate with the cell surface or extracellular matrix components making it difficult to detect in the medium. The insensitivity of the secreted, larger form to Endo H is a common feature of secreted glycoproteins. During transit through the Golgi apparatus, the Asn-linked carbohydrate moiety is modified by a series of specific glycosidases (reviewed in Kornfeld, R. and Kornfeld, S., (1985) *Annu. Rev. Biochem* 54:631-664; Tarentino, A.L. et al., (1989) *Methods Cell Biol.* 32:111-139). These modifications convert the structure from the immature "high mannose" to the mature "complex" type. At one step in this process, a Golgi enzyme, α -

mannosidase II, removes two mannose residues from the complex rendering it insensitive to Endo H (Kornfeld, R. and Kornfeld, S., (1985) *Annu. Rev. Biochem* 54:631-664).

Based on the observed molecular masses of the processed forms of mouse and chick *Shh*, the predicted secondary proteolytic cleavage site would be located near the border of the sequences encoded by the second and third exons. Interestingly, this region marks the end of the most highly related part of the *hedgehog* proteins. The amino terminal (smaller) form would contain the most highly conserved portion of the protein. In fact, the amino acids encoded by exons one and two (exclusive of sequences upstream of the putative signal peptide cleavage sites) share 69% identity between Dros-HH and mouse *Shh*, and 99% identity between chick and mouse *Shh*. Amino acid identity in the region encoded by the third exon is much lower 30% mouse to Drosophila and 71% mouse to chick (Echelard, Y. et al., (1993) *Cell* 75:1417-1430). Therefore, the two processed forms of *Shh* may have conserved as well as divergent signaling activities separated into distinct coding exons in the *Shh* gene. Furthermore, the observation that some unprocessed protein is secreted by *Xenopus* oocytes and cos cells raises the possibility that it may have a separate function.

The biochemical behavior of mouse *Shh* appears to be quite similar to that described for the Drosophila *Hedgehog* (Dros-HH) protein (Lee, J.L. et al., (1992) *Cell* 71:33-50; Tabata, T. et al., (1992) *Genes & Dev.* 6:2635-2645). *In vitro* translation of Dros-HH mRNA, in the presence of microsomes, revealed products with molecular masses corresponding to full length protein, as well as to the product expected after cleavage of the predicted internal (Type II) signal peptide (Lee, J.L. et al., (1992) *Cell* 71:33-50). Interestingly, no additional, processed forms were observed. However, such forms could have been obscured by breakdown products migrating between 20 and 30 kDa. When an RNA encoding a form of the protein lacking the carboxy-terminal 61 amino acids was translated, no breakdown products were seen, but there is still no evidence of the proteolytic processing observed with mouse *Shh*. A similar phenomenon has been observed in these experiments. A reduction in the extent of proteolytic processing is seen when a mouse *Shh* protein lacking 10 carboxy-terminal amino acids is translated *in vitro* or expressed in cos cells (data not shown). This suggests that sequences at the carboxy termini of Dros-HH proteins act at a distance to influence the efficiency of processing.

In vivo, processing of Dros-HH has been demonstrated (Tabata, T. et al., (1992) *Genes & Dev.* 6:2635-2645). Immunoblots of lysates from Schneider cells transfected with a *hh* expression vector reveal two smaller molecular weight forms similar to those described for mouse *Shh*. These products were also detected in extracts of larvae and imaginal discs derived from flies expressing a heat shock inducible *hh* construct. Thus, it is clear that there are also several distinct forms of Dros-HH proteins.

(vi) *Hedgehog Signaling*

In order to satisfy the criteria for intercellular signaling, *hedgehog* proteins must be detected outside of their domains of expression. This has been clearly demonstrated for Dros-HH. Using an antiserum raised against nearly full length Dros-HH protein, Tabata and Kornberg (Tabata, T. and Kornberg, T.B., (1992) *Cell* 76:89-102) detect the protein in stripes that are slightly wider than the RNA expression domains in embryonic segments, and just anterior to the border of the RNA expression domain in wing imaginal discs. Similarly, Taylor, et. al., (1993) *Mech. Dev.* 42:89-96, detected Dros-HH protein in discrete patches within cells adjacent to those expressing *hh* RNA in embryonic segments using an antiserum directed against an amino-terminal portion of Dros-HH which, based on the proteolytic processing data (Tabata, T. et al., (1992) *Genes & Dev.* 6:2635-2645), is not likely to recognize the carboxyl cleavage product.

The detection of Dros-HH beyond cells expressing the *hh* gene is consistent with the phenotype of *hh* mutants. In these animals, cellular patterning in each embryonic parasegment is disrupted resulting in an abnormal cuticular pattern reminiscent of that seen in *wg* mutants. Further analysis has revealed that the loss of *hh* gene function leads to loss of *wg* expression in a thin stripe of cells just anterior to the *hh* expression domain (Ingham, P.W. and Hidalgo, A., (1993) *Development* 117:283-291). This suggests that Dros-HH acts to maintain *wg* expression in neighboring cells. The observation that ubiquitously expressed Dros-HH leads to ectopic activation of *wg* supports this model (Tabata, T. and Kornberg, T.B., (1992) *Cell* 76:89-102). In addition to these genetic studies, there is also indirect evidence that Dros-HH acts at a distance from its site of expression to influence patterning of the epidermis (Heemskerk, J. and DiNardo, S., (1994) *Cell* 76:449-460).

The apparent effect of Dros-HH on neighboring cells, as well as on those located at a distance from the site of *hh* expression is reminiscent of the influence of the notochord and floor plate on the developing vertebrate CNS, and of the ZPA in the limb. The notochord (a site of high level *Shh* expression) induces the formation of the floor plate in a contact dependent manner, while the notochord and floor plate (another area of strong *Shh* expression) are both capable of inducing motoneurons at a distance (Placzek, M. et al., (1993) *Development* 117:205-218; Yamada, T. et al., (1993) *Cell* 73:673-686).

Moreover ZPA activity is required not only for patterning cells in the extreme posterior of the limb bud where *Shh* is transcribed, but also a few hundred microns anterior of this zone. Several lines of evidence indicate that *Shh* is able to induce floor plate (Echelard, Y. et al., (1993) *Cell* 75:1417-1430; Roelink, H. et al., (1994) *Cell* 76:761-775) and mediate the signaling activity of the ZPA (Riddle, R.D. et al., (1993) *Cell* 75:1401-1416). Since it has been shown that *Shh* is cleaved, it can be speculated that the processed peptides may have

115

distinct activities. The smaller amino terminal form, which appears to be more poorly secreted, less stable or retained at the cell surface or in the extracellular matrix, may act locally. In contrast, the larger carboxy terminal peptide could possibly function at a distance. In this way, *Shh* peptides may mediate distinct signaling functions in the vertebrate embryo.

5

Example 7

Sonic hedgehog and Fgf-4 act through a signaling cascade and feedback loop to integrate growth and patterning of the developing limb bud

(i) Experimental Procedures

10 *Cloning of Chicken Fgf-4 and Bmp-2*

A 246 bp fragment of the chicken *Fgf-4* gene was cloned by PCR from a stage 22 chicken limb bud library. Degenerate primers were designed against previously cloned *Fgf-4* and *Fgf-6* genes: fgf5' (sense) AAA AGC TTT AYT GYT AYG TIG GIA THG G (SEQ ID No:38) and fgf3' (antisense) AAG AAT TCT AIG CRT TRT ART TRT TIG G (SEQ ID No:39). Denaturation was at 94°C for 2 min, followed by 30 cycles of 94°C for 30 sec, 50°C for 60 sec, and 72°C for 30 sec, with a final extension at 72°C for 5 min. The PCR product was subcloned into the Bluescript SK+ vector. A clone was sequenced and confirmed as *Fgf-4* by comparison with previously published *Fgf-4* genes and a chicken *Fgf-4* gene sequence kindly provided by Lee Niswander.

BMP-related sequences were amplified from a stage 22 posterior limb bud cDNA library prepared in Bluescript using primers and conditions as described by Basler, et al. (1993). Amplified DNAs were cloned and used to screen a stage 22 limb bud library prepared in λ -Zap (Stratagene). Among the cDNAs isolated was chicken *Bmp-2*. Its identity was confirmed by sequence comparison to the published clones (Francis, et al., (1994) *Development* 120:209-218) and by its expression patterns in chick embryos.

Chick Surgeries and Recombinant Retroviruses

30 All experimental manipulations were performed on White Leghorn chick embryos (S-SPF) provided by SPAFAS (Norwich, Conn). Eggs were staged according to Hamburger and Hamilton (1951) *J. Exp. Morph.* 88:49-92.

Viral supernatants of *Sonic*/RCAS-A2 or a variant containing an influenza hemagglutinin epitope tag at the carboxyl terminus of the *hedgehog* protein (*Sonic*7.1/RCAS-A2, functionally indistinguishable from *Sonic*/RCAS-A2), were prepared as described (Hughes, et al., (1987) *J. Virol.* 61:3004-13; Fekete and Cepko, (1993) *Mol. & Cell. Biol.* 13:2604-13; Riddle, et al., (1993) *Cell* 75:1401-16). For focal injections the right wings of

35

stage 18-21 embryos were transiently stained with Nile blue sulfate (0.01 mg/ml in Ringer's solution) to reveal the AER. A trace amount of concentrated viral supernatant was injected beneath the AER.

The AER was removed using electrolytically sharpened tungsten wire needles. Some embryos had a heparin-acrylic bead soaked in *FGF-4* solution (0.8 mg/ml; a gift from Genetics Institute) or PBS stapled to the limb bud with a piece of 0.025mm platinum wire (Goodfellow, Cambridge UK) essentially as described by Niswander et al, (1993) *Cell* 75:579-87.

Limbs which were infected with *Sonic*/RCAS virus after AER removal were infected over a large portion of the denuded mesoderm to ensure substantial infection. Those embryos which received both an *Fgf-4* soaked bead and virus were infected only underneath the bead.

In Situ Hybridizations and Photography

Single color whole mount in situ hybridizations were performed as described (Riddle, et al., (1993) *Cell* 75:1401-16). Two color whole mount in situ hybridizations were performed essentially as described by Jowett and Lettice (1994) *Trends Genet.* 10:73-74. The second color detection was developed using 0.125mg/ml magenta-phos (Biosynth) as the substrate. Radioactive in situ hybridizations on 5µm sections was performed essentially as described by Tessarollo, et al. (1992) *Development* 115:11-20.

The following probes were used for whole mount and section in situ hybridizations: *Sonic*: 1.7kb fragment of pHH2 (Riddle, et al., (1993) *Cell* 75:1401-16). *Bmp-2*: 1.5 kb fragment encoding the entire open reading frame. *Fgf-4*: 250 bp fragment described above. *Hox d-11*: a 600 bp fragment, *Hoxd-13*: 400 bp fragment both including 5' untranslated sequences and coding sequences upstream of the homeobox. RCAS: 900 bp *Sall*-*Cla*I fragment of RCAS (Hughes et al., (1987) *J Virol.* 61:3004-12).

(ii) Relationship of Sonic to Endogenous Bmp-2 and Hoxd Gene Expression

The best candidates for genes regulated by *Sonic* in vivo are the distal members of the *Hoxd* gene cluster, *Hoxd-9* through *-13*, and *Bmp-2*. Therefore, the relationships of the expression domains of these genes in a staged series of normal chick embryos were analyzed. *Hoxd-9* and *Hoxd-10* are expressed throughout the presumptive wing field at stage 16 (Hamburger and Hamilton, (1951) *J. Exp. Morph.* 88:49-92), prior to the first detectable expression of *Sonic* at early stage 18. *Hoxd-11* expression is first detectable at early stage 18, the same time as *Sonic*, in a domain coextensive with *Sonic*. Expression of *Hoxd-12* and *Hoxd-13* commence shortly thereafter. These results suggest that *Sonic* might normally

induce, directly or indirectly, the expression of only the latter three members of the cluster, even though all five are nested within the early limb bud.

As limb outgrowth proceeds *Sonic* expression remains at the posterior margin of the bud. In contrast the *Hoxd* gene expression domains, which are initially nested posteriorly around the *Sonic* domain, are very dynamic and lose their concentric character. By stage 23 the *Hoxd-11* domain extends anteriorly and distally far beyond that of *Sonic*, while *Hoxd-13* expression becomes biased distally and displaced from *Sonic*.

While it is not clear whether *Bmp-2* is expressed before *Sonic* (see Francis et. al., (1994) *Development* 120:209-218) *Bmp-2* is expressed in a mesodermal domain which apparently overlaps and surrounds that of *Sonic* at the earliest stages of *Sonic* expression. As the limb bud develops, the mesodermal expression of *Bmp-2* remains near the posterior limb margin, centered around that of *Sonic*, but in a larger domain than *Sonic*. This correspondence between *Sonic* and *Bmp-2* expression lasts until around stage 25, much longer than the correspondence between *Sonic* and *Hoxd* gene expression. After stage 25 *Bmp-2* expression shifts distally and is no longer centered on *Sonic*.

(iii) Relationship of *Sonic* to Induced *Bmp-2* and *Hoxd* Gene Expression

The fact that the expression domains of the *Hoxd* genes diverge over time from that of *Sonic hedgehog* implies that *Sonic* does not directly regulate their later patterns of expression. This does not preclude the possibility that the later expression domains are genetically downstream of *Sonic*. If this were the case, exogenously expressed *Sonic* would be expected to initiate a program of *Hoxd* gene expression which recapitulates that seen endogenously. Therefore, the spatial distribution of *Hoxd* gene expression at various times following *Sonic* misexpression was compared. The anterior marginal mesoderm of early bud (Stage 18-20) wings was injected at a single point under the AER with a replication competent virus that expresses a chicken *Sonic* cDNA. Ectopic *Sonic* expressed by this protocol leads to both anterior mesodermal outgrowth and anterior extension of the AFR.

The *Sonic* and *Hoxd* gene expression domains in the infected limbs were analyzed in sectioned and intact embryos. Viral *Sonic* message is first detected approximately 18 hours after infection at the anterior margin, at the same time as, and approximately coextensively with, induced *Hoxd-11*. This suggests that *Sonic* can rapidly induce *Hoxd-11* expression and that the lag after injection represents the time required to achieve *Sonic* expression. By 35 hours post infection distal outgrowth of infected cells combined with lateral viral spread within the proliferating cells leads to viral expression in a wedge which is broadest at the distal margin and tapers proximally. By this time, *Hoxd-11* expression has expanded both antero-proximally and distally with respect to the wedge of *Sonic*-expressing cells, into a domain which appears to mirror the more distal aspects of the endogenous *Hoxd-11* domain.

Weak *Hoxd-13* expression is also detected at 35 hours in a subset of the *Sonic* expressing domain at its distal margin. 51 hours after infection the relationship of *Sonic* and *Hoxd-11* expression is similar to that seen at 35 hours, while the induced *Hoxd-13* expression has reached wild type levels restricted to the distal portions of the ectopic growth. Thus the ectopic *Hoxd* expression domains better reflect the endogenous patterns of expression than they do the region expressing *Sonic*. This suggests that there are multiple factors regulating *Hoxd* expression but their actions lie downstream of *Sonic*.

Since the endogenous *Bmp-2* expression domain correlates well with that of *Sonic*, and *Bmp-2* is induced by ZPA grafts, it was looked to see if *Bmp-2* is also induced by *Sonic*. *Bmp-2* is normally expressed in two places in the early limb bud, in the posterior mesoderm and throughout the AER (Francis, et al., (1994) *Development* 120:209-218). In injected limb buds additional *Bmp-2* expression is seen in both the anterior mesoderm and in the anteriorly extended AER. The domain of *Bmp-2* expression is slightly more restricted than that of viral expression, suggesting a delay in *Bmp-2* induction. *Bmp-2* expression in both the mesoderm and ectoderm is thus a downstream target of *Sonic* activity in the mesoderm. In contrast to the expression domains of the *Hoxd* genes, the endogenous and ectopic *Bmp-2* expression domains correlate well with that of *Sonic*. This suggests that *Bmp-2* expression is regulated more directly by *Sonic* than is expression of the *Hoxd* genes.

(iv) *The AER and Competence to Respond to Sonic*

Ectopic activation of *Hoxd* gene expression is biased distally in virally infected regions, suggesting that ectodermal factors, possibly from the AER, are required for *Hoxd* gene induction by *Sonic*. To test this, *Sonic* virus was injected into the proximal, medial mesoderm of stage 21 limb buds, presumably beyond the influence of the AER. Although the level of *Sonic* expression was comparable to that observed in distal injections, proximal misexpression of *Sonic* did not result in ectopic induction of the *Hoxd* genes or *Bmp-2*, nor did it result in any obvious morphological effect (data not shown). The lack of gene induction following proximal misexpression of *Sonic* suggests that exposure to *Sonic* alone is insufficient to induce expression of these genes.

This was tested more rigorously by injection of *Sonic* virus into the anterior marginal mesoderm of stage 20/21 limb buds after the anterior half of the AER had been surgically removed. Embryos were allowed to develop for a further 36 to 48 hours before harvesting. During this time the AER remaining on the posterior half of the limb bud promotes almost wild type outgrowth and patterning of the bud. Gene expression was monitored both in sectioned and intact embryos. In the presence of the AFR, *Sonic* induces both anterior mesodermal proliferation and expression of *Hoxd-11*, *Hoxd-13* and *Bmp-2*. In the absence of the overlying AER, *Sonic* does not induce either mesodermal proliferation or expression of

these genes above background. Signals from the AER are thus required to allow both the proliferative and patterning effects of *Sonic* on the mesoderm.

Since application of FGF protein can rescue other functions of the AER such as promoting PD outgrowth and patterning, it was sought to determine whether FGFs might also promote mesodermal competence to respond to *Sonic*. FGF-4-soaked beads were stapled to AER-denuded anterior mesoderm which was infected with *Sonic* virus. Gene expression and mesodermal outgrowth were monitored as described previously. In the presence of both *Sonic* virus and FGF-4 protein, *Hoxd-11*, *Hoxd-13* and *Bmp-2* expression are all induced. The expression levels of the induced genes are similar to or greater than the endogenous expression levels, and are equivalent in magnitude to their induction in the presence of the AER. Thus *Fgf-4* can induce the competence of the mesoderm to respond to *Sonic*.

Sonic alone is insufficient to induce either gene expression or mesodermal proliferation in the absence of the AER, while the combination of *Sonic* and FGF-4 induces both proliferation and gene expression. It was then asked whether FGF-4 alone has any effect on gene induction or mesodermal proliferation. Application of FGF-4 in the absence of *Sonic* virus does not induce *Hoxd* or *Bmp-2* gene expression above control levels, however FGF-4 alone induces mesodermal outgrowth. These results suggest that mesodermal gene activation requires direct action of *Sonic* on the mesoderm and that proliferative response to *Sonic* is indirect, due to the induction of FGFs.

(v) *Sonic* Induces Polarized *Fgf-4* Expression in the AER

Fgf-4 is expressed in a graded fashion in the AER of the mouse limb bud, with maximal expression at the posterior region of the AER tapering to undetectable levels in the anterior ridge (Niswander and Martin, (1992) *Development* 114:755-68). Therefore, it was appropriate to investigate whether *Fgf-4* is asymmetrically expressed in the chick AER, and whether its expression is induced by *Sonic*. A fragment of the chicken *Fgf-4* gene was cloned from a stage 22 chicken limb library by PCR using degenerate primers designed from mouse *Fgf-4* and *Xenopus e-Fgf* sequence; based on information provided by L. Niswander and G. Martin. Assignment of gene identity was based on primary sequence as well as comparison of expression patterns with that of murine *Fgf-4* (Niswander and Martin, (1992) *Development* 114:755-68). Whole mount in situ hybridization analysis showed strong limb expression of chick *Fgf-4* in the AER. *Fgf-4*, like *Bmp-2*, is expressed all the way to the posterior border of the AER, but its anterior domain ends before the morphological end of the AER creating a posterior bias that has also been observed by Niswander et al., (1994) *Nature* (in press). Expression is first detected in the distal AER at about stage 18. As outgrowth proceeds the posterior bias develops. Expression peaks around stage 24/25 and then fades by stage 28/29.

120

The expression domain of *Fgf-4* becomes posteriorly biased as *Sonic* is expressed in the posterior mesoderm. This observation is consistent with *Sonic* influencing the expression of *Fgf-4* in the posterior AER. To test the effect of *Sonic* on *Fgf-4* expression in the AER, stage 18-20 embryos were infected with *Sonic* virus in a single point at their anterior margin beyond the anterior limit of the AER. The embryos were harvested one to two days later, when an extension of the anterior AER became apparent. The expression of *Fgf-4* was analyzed by in situ hybridization. *Fgf-4* expression is induced in the anteriormost segment of the AER, in a region which is discontinuous with the endogenous expression domain, and overlies the domain of viral *Sonic* infection. This result contrasts with the *Bmp-2* expression induced in the extended AER, which is always continuous with the endogenous expression domain. The asymmetry of the induced *Fgf-4* expression indicates that *Sonic* polarizes the extended AER, much as a ZPA graft does (Maccabe and Parker, (1979) *J. Embryol. Exp. Morph.* 53:67-73). Since FGFs by themselves are mitogenic for limb mesoderm, these results are most consistent with *Sonic* inducing distal proliferation indirectly, through the induction of mitogens in the overlying AER.

(vi) *Reciprocal Regulation of Sonic by Fgf-4*

Sonic thus appears to be upstream of *Fgf-4* expression in the AER. However, since the AER is required to maintain polarizing activity in the posterior mesoderm (Vogel and Tickle, (1993) *Development* 19:199-206; Niswander et al., (1993) *Cell* 75:579-87), *Sonic* may also be downstream of the AER. If *Sonic* is regulated by the AER and the AER by *Sonic*, this would imply that they are reinforcing one another through a positive feedback loop.

To test whether the AER dependence of ZPA activity is controlled at the level of transcription of the *Sonic* gene, *Sonic* expression following removal of the AER from the posterior half of the limb bud was assayed. *Sonic* expression is reduced in an operated limb compared to the contralateral control limb within ten hours of AER removal, indicating that *Sonic* expression is indeed AER dependent. The dependence of *Sonic* expression on signals from the AER suggests that one of the functions of the AER is to constrain *Sonic* expression to the more distal regions of the posterior mesoderm.

In addition to their mitogenic and competence-inducing properties, FGFs can also substitute for the AER to maintain the ZPA. In order to test whether FGFs can support the expression of *Sonic*, beads soaked in FGF-4 protein were stapled to the posterior-distal tips of limb buds after posterior AER removal. Embryos were assayed for *Sonic* expression approximately 24 hours later, when *Sonic* expression is greatly reduced in operated limb buds which had not received an FGF-4 bead. Strong *Sonic* expression is detectable in the posterior mesoderm, slightly proximal to the bead implant, and reflecting the normal domain of *Sonic*

121

expression seen in the contralateral limb. With the finding that FGF-4 can maintain *Sonic* expression, the elements required for a positive feedback loop between *Sonic* expression in the posterior mesoderm and *Fgf-4* expression in the posterior AER are established (see also Niswander et al. (1994) *Nature* (in press)).

5 The induction of *Bmp-2* expression by *Sonic* requires signals from the AER, and its domain correlates over time with that of *Sonic*. Therefore, it was interesting to learn if the continued expression of *Bmp-2* also requires signals from the AER, and if so, whether they could be replaced by FGF-4. To test this, *Bmp-2* expression following posterior AER removal, and following its substitution with an FGF-4 bead was assayed. *Bmp-2* expression
10 fades within hours of AER removal, and can be rescued by FGF-4. These data indicate that the maintenance of *Bmp-2* expression in the posterior mesoderm, like that of *Sonic*, is dependent on signals from the AER, which are likely to be FGFs.

(vii) *The Mesodermal Response to Sonic*

15 It has been found that only mesoderm underlying the AER is responsive to *Sonic*, apparently because the AER is required to provide competence signals to the limb mesoderm. *Fgf-4*, which is expressed in the AER, can substitute for the AER in this regard, and thus might act in combination with *Sonic* to promote *Hoxd* and *Bmp-2* gene expression in the mesoderm. FGFs may be permissive factors in a number of instructive pathways, as they are
20 also required for activins to pattern *Xenopus* axial mesoderm (Cornell and Kimelman, (1994) *Development* 120:2187-2198; LaBonne and Whitman, (1994) *Development* 120:463-472).

 The induction of *Hoxd* and *Bmp-2* expression in response to *Sonic* and FGF-4 in the absence of an AER suggests that the mesoderm is a direct target tissue of *Sonic* protein. Since *Sonic* can induce *Fgf-4* expression in the AER, it follows that *Sonic* also acts indirectly
25 on the mesoderm through the induction of competence factors in the AER.

(viii) *Downstream Targets and a Cascade of Signals Induced by Sonic*

 The five AbdB-like *Hoxd* genes, *Hoxd-9* through *-13*, are initially expressed in a nested pattern centered on the posterior of the limb bud, a pattern which suggests they might
30 be controlled by a common mechanism (Dolle, et al., (1989) *Cell* 75:431-441; Izpisua-Belmonte, et al., (1991) *Nature* 350:585-9). The analysis of the endogenous and induced domains of *Hoxd* gene expression suggests that *Sonic* normally induces expression of *Hoxd-11*, *-12* and *-13*. In contrast it was found that *Hoxd-9* and *-10* expression initiate before *Sonic* mRNA is detectable. This implies that at least two distinct mechanisms control the initiation
35 of *Hoxd* gene expression in the wing bud, only one of which is dependent on *Sonic*.

 Several observations suggest that the elaboration of the *Hoxd* expression domains is not controlled directly by *Sonic*, but rather by signals which are downstream of *Sonic*. The *Hoxd* expression domains rapidly diverge from *Sonic*, and evolve into several distinct

subdomains. Moreover these subdomains appear to be separately regulated, as analysis of the murine *Hoxd-11* gene promoter suggests that it contains independent posterior and distal elements (Gerard, et al., (1993) *Embo. J.* 12:3539-50). In addition, although initiation of *Hoxd-11* through -13 gene expression is dependent on the AER, their expression is maintained following AER removal (Izpisua-Belmonte, et al., (1992) *Embo. J.* 11:1451-7). As *Sonic* expression fades rapidly under similar conditions, this implies that maintenance of *Hoxd* gene expression is independent of *Sonic*. Since ectopic *Sonic* can induce a recapitulation of the *Hoxd* expression domains in the limb, it can be concluded that although indirect effectors appear to regulate the proper patterning of the *Hoxd* expression domains, they are downstream of *Sonic*. Potential mediators of these indirect effects include *Bmp-2* in the mesoderm and *Fgf-4* from the AER.

In contrast to the *Hoxd* genes, *Bmp-2* gene expression in the posterior limb mesoderm appears to be continually regulated by *Sonic*. It was found that both endogenous and ectopic *Bmp-2* expression correspond to that of *Sonic*. Furthermore, continued *Bmp-2* expression is dependent on the AER and can be rescued by FGF-4. It is likely that this is an indirect consequence of the fact that *Sonic* expression is also maintained by the AER and can be rescued by FGF-4. In fact, *Bmp-2* expression might be a direct response of cells to secreted *Sonic* protein. The differences between *Bmp-2* and *Hoxd* gene expression suggest that multiple pathways downstream of *Sonic* regulate gene expression in the mesoderm.

Bmp-2 itself is a candidate for a secondary signaling molecule in the cascade of patterning events induced by *Sonic*. *Bmp-2* is a secreted molecule of the TGF- β family and its expression can be induced by *Sonic*. This appears to be an evolutionarily conserved pathway, as Dros-HH, the Drosophila homolog of *Sonic*, activates the expression of *dpp*, the homolog of *Bmp-2*, in the eye and wing imaginal discs (Heberlein, et al., (1993) *Cell* 75:913-26; Ma, et al., (1993) *Cell* 75:927-38; Tabata and Kornberg, (1994) *Cell* 76:89-102). Expression of Dros-HH is normally confined to the posterior of the wing disc. Ectopic expression of Dros-HH in the anterior of the disc results in ectopic expression of *dpp* and ultimately in the duplication of wing structure with mirror image symmetry (Bassler and Struhl, (1994) *Nature* 368:208-214). This effect is strikingly parallel to the phenotypic results of ectopic expression of *Sonic* in the chick limb.

(ix) Regulation of *Sonic* Expression

Sonic expression is activated in the posterior of the limb bud very early during mesodermal outgrowth (Riddle et al., (1993) *Cell* 75:1401-16). The factors which initiate this localized expression are not yet identified but ectopic expression of *Hoxb-8* at the anterior margin of the mouse limb bud results in the activation of a second domain of *Sonic* expression under the anterior AER (Charité et al., (1994) *Cell* 78:589-601). Since retinoic acid is known to be able to induce the expression of *Hoxb-8* and other *Hox* genes in vitro

(Mavilio et al., (1988) *Differentiation* 37:73-79) it is possible that endogenous retinoic acid acts to make cells competent to express *Sonic* by inducing expression of upstream *Hox* genes, either in the very early limb bud or in the flank prior to the limb bud formation.

Several lines of evidence suggest that once induced *Sonic* expression is dependent on signals from the posterior AER. Following its initiation in the posterior limb mesoderm, the *Sonic* expression domain moves distally as the limb bud grows out, always remaining subjacent to the AER. Similarly, *Sonic* expression can also be induced on the anterior margin of the limb bud by implantation of a retinoic acid bead, but the induced ectopic expression is limited to the mesoderm directly underlying the AER (Riddle, et al., (1993) *Cell* 75:1401-16). In addition, ZPA activity fades rapidly following removal of the AER (Niswander, et al., (1993) *Cell* 75:579-87; Vogel and Tickle, (1993) *Development* 119:199-206), and ZPA grafts only function when placed in close proximity to the AER (Tabin, (1991) *Cell* 66:199-217; Tickle, (1991) *Development Supp.* 1:113-21). The observation that continued *Sonic* expression depends on signals from the posterior AER reveals the mechanism underlying these observations.

The reliance of *Sonic* expression on AER-derived signals suggests an explanation for the distal shift in *Sonic* expression during limb development (Riddle et al., (1993) *Cell* 75:1401-16). Signals from the AER also promote distal outgrowth of the mesodermal cells of the progress zone, which in turn results in the distal displacement of the AER. Hence, as maintenance of *Sonic* expression requires signals from the AER, its expression domain will be similarly displaced.

It was found that replacement of the AER with *FGF-4* soaked beads results in the maintenance of *Sonic* expression. This result is consistent with the previous findings that ZPA activity can be maintained in vivo and in vitro by members of the FGF family (Anderson, et al., (1993) *Development* 117:1421-33; Niswander et al., (1993) *Cell* 75:1401-16; Vogel and Tickle, (1993) *Development* 119:199-206). Since *Fgf-4* is normally expressed in the posterior AER, these results suggest that *Fgf-4* is the signal from the ectoderm involved in maintaining *Sonic* expression.

(x) *Sonic* and Regulation and Maintenance of the AER

Sonic can induce anterior extensions of the AER which have an inverted polarity relative to the endogenous AER. This polarity is demonstrated by examining the expression of two markers in the AER. In normal limbs *Bmp-2* is expressed throughout the AER, while *Fgf-4* is expressed in the posterior two thirds of the AER. In the extended AER resulting from ectopic *Sonic* expression, *Bmp-2* is again found throughout the AER, while *Fgf-4* expression is biphasic, found at either end of the AER, overlying the anterior and posterior mesodermal domains expressing *Sonic*. These results are consistent with previous observations that antero-posterior polarity of the AER appears to be regulated by the

underlying mesoderm, and that ZPA grafts lead to the induction of ectopic, polarized AER tissue (Maccabe and Parker, (1979) *J. Embryol. Exp. Morph.* 53:67-73). Our results also suggest that the normal AP polarity of the AER is a reflection of endogenous *Sonic* expression. The induced AER is sufficient to promote complete PD outgrowth of the induced structures (Riddle et al., (1993) *Cell* 75:1401-16). Hence whatever factors are necessary to maintain the AER are also downstream of *Sonic*.

(xi) *A Positive Feedback Loop Between Sonic and Fgf-4*

The induction of *Fgf-4* expression by *Sonic* in the ectopic AER, and the maintenance of *Sonic* expression by FGF-4 suggest that *Sonic* and *Fgf-4* expression are normally sustained by a positive feedback loop. Such a feedback loop would allow the coordination of mesodermal outgrowth and patterning. This coordination is possible because *Sonic* patterns mesodermal tissue and regulates *Fgf-4* expression, while FGF-4 protein induces mesodermal proliferation and maintains *Sonic* expression. Moreover mesodermal tissue can only be patterned by *Sonic* in the context of a competence activity provided by FGF-4. Thus patterning is always coincident with proliferation.

It remains possible that exogenously applied *Fgf-4* might be mimicking the activity of a different member of the FGF family. For example, *Fgf-2* is expressed in the limb mesoderm and the AER (Savage et al., (1993) *Development Dynamics* 198:159-70) and has similar effects on limb tissue as *Fgf-4* (Niswander and Martin, (1993) *Nature* 361:68-71; Niswander, et al., (1993) *Cell* 75:579-87; Riley, et al., (1993) *Development* 118:95-104; Fallon, et al., (1994) *Science* 264:104-7).

(xii) *Coordinated Regulation of Limb Outgrowth and Patterning*

Patterning and outgrowth of the developing limb are known to be regulated by two major signaling centers, the ZPA and AER. The identification of *Sonic* and FGFs as molecular mediators of the activities of the ZPA and AER has allowed for dissociation of the activities of these signaling centers from their regulation, and investigation of the signaling pathways through which they function.

The results presented above suggest that the ability of cells to respond to *Sonic* protein is dependent on FGFs produced by the AER. It was also found that *Sonic* induces a cascade of secondary signals involved in regulating mesodermal gene expression patterns. In addition evidence was found for a positive feedback loop initiated by *Sonic*, which maintains expression of *Sonic* in the posterior mesoderm and *Fgf-4* in the AER. The feedback loop described suggests a mechanism whereby outgrowth and patterning along the AP and PD axes of the limb can be coordinately regulated.

125

The results described above further suggest that *Sonic* acts as a short range signal which triggers a cascade of secondary signals whose interplay determines the resultant pattern of structures. The data suggest a number of inductive pathways that can be combined to generate a model (Figure 14) which describes how *Sonic*, in coordination with the AER, acts to pattern mesodermal tissues along the anterior-posterior limb axis, while simultaneously regulating proximal-distal outgrowth.

Following its induction, *Sonic* signals to both the limb ectoderm and mesoderm. *Sonic* imposes a distinct polarity on the forming AER, including the posteriorly biased expression of *Fgf-4*, and the AER becomes dependent on continued *Sonic* expression. The mesoderm, as long as it is receiving permissive signals from the overlying ectoderm, responds to the *Sonic* signal by expressing secondary signaling molecules such as *Bmp-2* and by activating *Hoxd* genes. *Bmp-2* expression is directly dependent on continued *Sonic* expression, while the continued expression of the *Hoxd* genes, rapidly becomes *Sonic*. independent. In a reciprocal fashion, maintenance of *Sonic hedgehog* expression in the posterior mesoderm becomes dependent on signals from the AER. Since the factors expressed by the AER are not only required for the maintenance of *Sonic* expression and activity, but are also mitogenic, growth and patterning become inextricably linked. Coordination of limb development through interdependent signaling centers forces the AP and PD structures to be induced and patterned in tandem. The pathways elucidated herein thus provide a molecular framework for the controls governing limb patterning

Example 8

Sonic, BMP-4, and Hox Gene Expression Suggest a Conserved Pathway in Patterning the Vertebrate and Drosophila Gut

(i) Experimental Procedure

In Situ Hybridization and Photography

BMP probes were isolated using primers designed to amplify members of the TGF- and BMP families (Basler, K. et al., (1993) *Cell* 73:687-702, eight independent 120 bp BMP fragments were amplified from a stage 22 chicken posterior limb bud plasmid cDNA library. These fragments were pooled and used to screen an unamplified stage 22 limb bud lambda zap cDNA library constructed as in Riddle et al., (1993) *Cell* 75:1401-16. Among the BMP related clones isolated were an approximately 1.9 kb cDNA clone corresponding to chicken *BMP-2* and an approximately 1.5 kb cDNA clone corresponding to chicken *BMP-4*. Both clones contain the entire coding regions. The *Sonic* clone was obtained as described in Riddle et al, (1993) *Cell* 75:1401-16. Digoxigenin-UTP labeled RNA probes were

transcribed as per Riddle et al., (1993) *Cell* 75:1401-16. Briefly, harvested chick embryos were fixed overnight in 4% paraformaldehyde, washed in PBS then processed for whole mount *in situ* hybridization methods are per Riddle et al., (1993) *Cell* 75:1401-16. Embryos were photographed from either ventral or dorsal surfaces under transmitted light using a Nikon zoom stereo microscope with Kodak Ektar 100 ASA film. Whole mount *in situ* hybridization embryos and viscera were processed for sectioning as described in Riddle et al., (1993) *Cell* 75:1401-16. 15-25 μ m transverse sections were air dried and photographed with brightfield or numarski optics using a Zeiss Axiophot microscope and Kodak Ektar 25 ASA film.

Chick Embryos and Recombinant Retroviruses

A retroviral vector engineered to express a full length cDNA of chicken *Sonic*, as in Riddle et al. (1993) *Cell* 75:1401-16, was injected unilaterally into stage 8-13 chicken embryos targeting the definitive endoderm at the mid-embryo level. At this stage the CIP has not formed and neither *Sonic* nor *BMP-4* are expressed in the region injected. Injections were performed on the ventral surface on embryos cultured with their ventral surface facing up (New, D.A.T. (1955) *Embryol. Exp. Morph.* 3:320-31. Embryos were harvested 18-28 hours after injection and prepared for whole mount *in situ* hybridization (see above description of *in situ* experiment), hybridized with *Sonic* or *BMP-4* digoxigenin labeled probes.

In Situ Hybridization with Hox Genes

Cloned cDNA of the chicken homologues of *Hoxa-9,-10,-11,-13; b-9, c-9,-10,-11; d-9,-10,-11,-12, and -13* were used to transcribe digoxigenen-UTP labeled riboprobes for whole mount *in situ* hybridization. Domestic chick embryos were harvested into PBS and eviscerated. The visceral organ block was fixed in 4% paraformaldehyde overnight and processed for whole mount *in situ* hybridization. Methods and photographic technique as described above.

(ii) Expression of Sonic and BMP-4 in Stage 13 Chick Embryos Determined by Whole Mount In Situ Hybridization

Chick gut morphogenesis begins at stage 8 (Hamberger and Hamilton, (1987) *Nutr.* 6:14-23 with a ventral in-folding of the anterior definitive endoderm to form the anterior intestinal portal (AIP) (Romanoff, A.L., (1960) *The Avian Embryo*, The Macmillan Co., NY. This lengthens posteriorly forming the foregut. A second wave of endodermal invagination is initiated posteriorly at stage 13, creating the caudal intestinal portal (CIP). The CIP extends

anteriorly forming the hindgut. *Sonic* expression, previously noted in the endoderm of the vertebrate gut (Riddle et al., (1993) *Cell* 75:1401-16; Echelard et al., (1993) *Cell* 75:1417-1430), is expressed early in a restricted pattern in the endodermal lips of the AIP and CIP. *Sonic* expression is detected in the endoderm of the AIP and CIP in pre gut closure stages. At later stages, stage 28 embryos, *Sonic* is expressed in the gut in all levels (fore-, mid-, and hind-gut) restricted to the endoderm. *Sonic* is known to be an important inductive signal in other regions of the embryo including the limb bud (Riddle et al., (1993) *Cell* 75:1401-16) and neural tube (Echelard et al., (1993) *Cell* 75:1417-1430; Kraus et al., (1994) *Cell* 75:1437-1444; Roelink et al., (1994) *Cell* 76:761-775). Since primitive gut endoderm is known to cause gut-specific mesodermal differentiation when combined with non-gut mesenchyme (Haffen et al., (1987) *Nutr.* 6:14-23), we speculated that *Sonic* might function as an inductive signal to the visceral mesoderm. A potential target gene for the action of *Sonic* was suggested by analogy to the *Drosophila* imaginal discs where Dros-HH, the homologue of vertebrate *Sonic*, activates the expression of the *TGF- β* related gene *dpp* in adjacent cells (Tabata abd Kornberg, (1994) *Cell* 76:89-102; Heberlein et al., (1993) *Cell* 75:913-926; Ma et al., (1993) *Cell* 75:913-926; Basler et al., (1993) *Cell* 73:687-702). There are two vertebrate homologues of *dpp*, *BMP-2* and *BMP-4*. The earliest detectable expression of *BMP-4* occurs simultaneously with the first observable expression of *Sonic* in the developing gut. *BMP-4* is expressed in a domain abutting *Sonic* at the AIP and the CIP, but is restricted to the adjacent ventral mesoderm. *BMP-4* gut expression persists into later stage embryos, stage 33 embryos, in the visceral mesoderm only. The tissue restricted expression of both genes is maintained in all stages studied. *BMP-2* is not expressed in the gut at the AIP or CIP, but is expressed in clusters of cells in the gut mesoderm in later stages, a pattern distinct from that of *BMP-4*.

(iii) *Ectopic Expression of Sonic Induces Ectopic Expression of BMP-4 in Mesodermal Tissues of the Developing Chick*

To test whether *Sonic* is capable of inducing *BMP-4* in the mesoderm we an ectopic expression system previously used to study the role of *Sonic* in limb development was utilized (Riddle et al., (1993) *Cell* 75:1401-16). A replication competent retrovirus engineered to express *Sonic* was injected unilaterally into the presumptive endoderm and visceral mesoderm at mid-embryo positions in stage 8-13 chick embryos *in vitro* (New, D.A.T. (1955) *Embryol. Exp. Morph.* 3:320-321). When embryos were examined by *in situ* hybridization 18-26 hours later, the normal wild type expression of *Sonic* is detected at the AIP, CIP, and in the midline (neural tube and notochord). Ectopic *Sonic* expression is present unilaterally on the left ventral surface. Also, wild type *Sonic* expression is seen in the floor plate of the neural tube and notochord. Ectopic expression is seen unilaterally in the

128

visceral endoderm, its underlying splanchnic mesoderm, and somatic mesoderm. *BMP-4* expression can be seen induced in the mesoderm at the site of injection, in addition to its normal expression in the mesoderm of the CIP. Wild type *BMP-4* expression is seen in the most dorsal aspects of the neural tube and symmetrical lateral regions adjacent to the neural tube. Induced *BMP-4* expression is present unilaterally in the splanchnic mesoderm at the site of *Sonic* viral injection, and not in the visceral endoderm.

Since *BMP-4* is, itself, a secreted protein, it could function as a secondary signal in an inductive cascade, similar to the signal cascades from Dros-HH to *dpp* in *Drosophila* imaginal discs (Tabata and Kornberg, (1994) *Cell* 76:89-102; Heberlein et al., (1993) *Cell* 75:913-926; Ma et al., (1993) *Cell* 75:913-926; Basler et al., (1993) *Cell* 73:687-702) and from *Sonic* to *BMP-2* in the limb bud. In the gut, *BMP-4* could act as a secondary signal either as part of a feedback loop to the endoderm or within the visceral mesoderm. This latter possibility is consistent with the finding that in mice homozygous for a deletion in the *BMP-4* gene, the ventral mesoderm fails to close.

(iv) Expression of Hox Genes in the Developing Chick Gut

There is a striking parallel between the apparent role of *Sonic* as an endoderm-to-mesoderm signal in early vertebrate gut morphogenesis and that of its *Drosophila* homologue, Dros-HH. Dros-HH (like *Sonic*) is expressed in the *Drosophila* gut endoderm from the earliest stages of morphogenesis (Taylor et al., (1993) *Mech. Dev.* 42:89-96). Its putative receptor, patched, is found in the visceral mesoderm implicating Dros-HH (like *Sonic*) in endodermal-mesodermal inductive interactions. This led to consideration whether other genes known to be involved in regulating *Drosophila* gut development might also play a role in regulating chick gut morphogenesis. Regionally specific pattern in *Drosophila* gut endoderm is regulated by a pathway involving restricted expression of homeotic genes in the mesoderm (McGinnis and Krumlauf, (1992) *Cell* 68:283-302). Although the basis for patterning the vertebrate gut is poorly understood, in several other regions of the embryo *Hox* genes have been implicated as key regulators of patterns. Vertebrate *Hox* genes are expressed in overlapping anteroposterior domains which correlate with structural boundaries in the developing hindbrain, vertebrae, and limbs (McGinnis and Krumlauf, (1992) *Cell* 68:283-302). Whole mount *in situ* hybridization was used to test whether these genes are also expressed in the developing vertebrate hindgut and whether their domains of expression correlate with morphologic borders of the chick gut.

Luminal gut differentiation creates three morphologically and physiologically distinct regions: fore-, mid-, and hind- gut. The fore-gut and hind-gut are the derivatives of the primitive gut tubes initiated at the AIP and CIP respectively. Ultimately these tubes meet and

fuse at the yolk stalk around stage 24-28. The midgut is formed from both foregut and hindgut primordia, just anterior and posterior to the yolk stalk.

The most posterior derivative of the hindgut is the cloaca, the common gut-urogenital opening. The rest of the hindgut develops into the large intestine. The midgut/hindgut border is demarcated by a paired tubal structure, the ceca (analogous to the mammalian appendix), which forms as budding expansions at the midgut/hindgut border at stage 19-20. Anterior to the ceca, the midgut forms the small intestine.

The expression pattern of the 5' members of the *Hox* gene clusters in the chick hindgut by whole mount *in situ* hybridization was studied. *Hox* gene expression patterns in the gut are dynamic. They are initially expressed (by stage 10) in broad mesodermal domains extending anteriorly and laterally. Later they become restricted. By stage 25, the Abd-B like genes of the *Hoxa* and *Hoxd* cluster are regionally restricted in their expression in hindgut mesoderm. The most anteriorly expressed gene, *Hoxa-9*, has an anterior border of expression within the mesoderm of the distal midgut (to a point approximating the distal third of the midgut length). Each successive gene within the A and D *Hox* clusters has a more posterior domain of expression. *Hoxa-10*, *Hoxd-9* and *Hoxd-10* are restricted in their expression to the ceca. *Hoxa-11* and *Hoxd-11* have an anterior limit of expression in the mid-ceca at the approximate midgut/hindgut boundary (Romanoff, A.L. (1960) *The Avian Embryo*, The Macmillan Co. NY). *Hoxd-12* has an anterior limit at the posterior border of the ceca and extends posteriorly throughout the hindgut to the cloaca. *Hoxa-13* and *Hoxd-13* are expressed in the most posteriorly restricted domain, in the ventral mesoderm surrounding the cloaca. *Hoxa-13* and *Hoxd-13* are the only Abd-B like genes which are also expressed within the gut endoderm, from the ceca to the cloaca.

The only member of the B or C *Hox* clusters which we found to be expressed in the hindgut is *Hoxc-9*. The expression of *Hoxc-9* overlaps with its paralogues *Hoxa-9* and *Hoxd-9* in the midgut mesoderm, but has a sharp posterior boundary, complementary to *Hoxa-11* and *Hoxd-11* in the mid-ceca.

The restricted expression of the Abd-B like *Hox* genes appear to demarcate the successive regions of the gut which will form the cloaca, the large intestine, the ceca, the mid-ceca at the midgut/hindgut border, and the lower portion of the midgut (perhaps identifying that portion of the midgut derived from the posterior gut tube3). Moreover, these molecular events presage regional distinctions. Expression of all *Hox* genes could be detected by stage 14, well before the hindgut lumen is closed (by stage 28) and is maintained in subsequent stages studied. Cytodifferentiation of the hindgut mesoderm and epithelium begins later, at stages 29-31 (Romanoff, A.L. (1960) *The Avian Embryo*, The Macmillan Co. NY).

These results suggest that specific *Hox* genes might be responsible for regulating morphogenesis of the gut. Consistent with this, there is an apparent homeotic alteration in

the gut of a transgenic mouse in which the anterior limit of expression of *Hoxc-8* is shifted rostrally: a portion of foregut epithelium mis-differentiates as midgut (Pollock and Bieberich, (1992) *Cell* 71:911-923).

5 (v) *Conservation in the Expression of Regulatory Genes Involved in the Formation of Vertebrate and Drosophila Gut*

There is an intriguing parallel between the expression patterns of *Sonic*, *BMP-4*, and the *Hox* genes in the vertebrate gut and those of their homologues during *Drosophila* gut morphogenesis (Figure 15). This conservation is of particular interest because in *Drosophila* the role played by these genes has been clarified genetically. Dros-HH (like its vertebrate homologue, *Sonic*) is expressed at the earliest stages in the gut endoderm and may be a signal to visceral mesoderm (Taylor et al., (1993) *Mech. Dev.* 42:89-96). Nothing is known directly of the relationship between Dros-HH expression and activation of expression of other genes in the *Drosophila* gut. However, in *Drosophila* imaginal discs, Dros-HH is known to activate the expression of *dpp* in a signaling cascade (Kraus et al., (1994) *Cell* 75:1437-1444; Heberlein et al., (1993) *Cell* 75:913-926; Ma et al., (1993) *Cell* 75:913-926; Basler et al., (1993) *Cell* 73:687-702). Later in gut development, the production of *dpp* in the mesoderm contributes to the regulation of the expression of homeotic genes in both the mesoderm and the endoderm (Bienz, M. (1994) *TIG* 10:22-26). *Drosophila* homeotic genes are expressed in the gut visceral mesoderm and their expression is known to determine the morphologic borders of the midgut. This involves proper induction of gene expression in the adjacent endoderm, one of the mediators of the interaction is *dpp* (Bienz, M. (1994) *TIG* 10:22-26). If Dros-HH is required for the ultimate activation of the homeotic genes in the *Drosophila* midgut, this would parallel the situation in the vertebrate limb bud where *Sonic* functions as an upstream activator of the *Hox* genes (Riddle et al., (1993) *Cell* 75:1401-1416), perhaps in a signaling cascade involving *BMP-2*.

The extraordinary conservation in the expression of regulatory genes in the vertebrate and *Drosophila* gut strongly suggests a conservation of patterning mechanisms. Pathways established by genetic studies in *Drosophila* provide direct insights into the molecular basis for the regionalization and morphogenesis of the vertebrate gut.

Example 9

Bacterially Expressed Hedgehog Proteins Retain Motorneuron-inducing Activity

35 Various fragments of the mouse *Shh* gene were cloned into the pET11D vector as fusion proteins with a poly(His) leader sequence to facilitate purification. Briefly, fusion genes encoding the mature M-*Shh* protein (corresponding to Cys-25 through Ser-437 of SEQ ID No. 11) or N-terminal containing fragments, and an N-terminal exogenous leader having

131

the sequence M-G-S-S-H-H-H-H-H-H-L-V-P-R-G-S-H-M (SEQ ID No: 47) were cloned in pET11D and introduced into *E. coli*. The poly(His)-*Shh* fusion proteins were purified using nickel chelate chromatography according to the vendor's instructions (Qiagen catalog 30210), and the poly(His) leader cleaved from the purified proteins by treatment with thrombin.

5 Preparations of the purified *Shh* proteins were added to tissue explants (neural tube) obtained from chicken embryos and cultured in a defined media (e.g., no serum). M-*Shh* protein was added to final concentrations of between 0.5pM to 5nM, and differentiation of the embryonic explant tissue to motoneuron phenotype was detected by expression of Islet-1 antigen. The bacterially produced protein was demonstrated to be active in the explant
10 cultures at concentrations as low as 5 to 50pM. An *Shh* polypeptide containing all 19kd of the amino terminal fragment and approximately 9kd of the carboxyl terminal fragment (see Example 6) displayed both motor neuron inducing activity and weak floor plate inducing activity, indicating that these activities likely reside with the N-terminal fragment.

15 All of the above-cited references and publications are hereby incorporated by reference.

Equivalents

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, numerous equivalents to the specific polypeptides, nucleic acids,
20 methods, assays and reagents described herein. Such equivalents are considered to be within the scope of this invention and are covered by the following claims.

132
SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:

(A) NAME: President and Fellows of Harvard College
(B) STREET: 124 Mt. Auburn Street
(C) CITY: Cambridge
(D) STATE: MA
(E) COUNTRY: USA
(F) POSTAL CODE (ZIP): 02138

(A) NAME: Imperial Cancer Research Technology Ltd.
(B) STREET: Sardinia Street
(C) CITY: London
(D) STATE: N/A
(E) COUNTRY: United Kingdom
(F) POSTAL CODE (ZIP): WC2A3NL

(ii) TITLE OF INVENTION: Vertebrate Embryonic Pattern-Inducing
Proteins and Uses Related Thereto

(iii) NUMBER OF SEQUENCES: 47

(iv) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE:

(v) CURRENT APPLICATION DATA:

APPLICATION NUMBER:

(vi) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER: US 08/176,427
(B) FILING DATE: 30-DEC-1993

(vi) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER: US 08/356,060
(B) FILING DATE: 14-DEC-1994

(viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: Vincent, Matthew P.
(B) REGISTRATION NUMBER: 36,709
(C) REFERENCE/DOCKET NUMBER: HMI-006PC

(ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: (617) 227-7400
(B) TELEFAX: (617) 227-5941

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1278 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

133

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

5

(A) NAME/KEY: CDS

(B) LOCATION: 1..1277

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

10

ATG	GTC	GAA	ATG	CTG	CTG	TTG	ACA	AGA	ATT	CTC	TTG	GTG	GGC	TTC	ATC	48
Met	Val	Glu	Met	Leu	Leu	Leu	Thr	Arg	Ile	Leu	Leu	Val	Gly	Phe	Ile	
1				5					10					15		

15

TGC	GCT	CTT	TTA	GTC	TCC	TCT	GGG	CTG	ACT	TGT	GGA	CCA	GGC	AGG	GGC	96
Cys	Ala	Leu	Leu	Val	Ser	Ser	Gly	Leu	Thr	Cys	Gly	Pro	Gly	Arg	Gly	
			20					25					30			

20

ATT	GGA	AAA	AGG	AGG	CAC	CCC	AAA	AAG	CTG	ACC	CCG	TTA	GCC	TAT	AAG	144
Ile	Gly	Lys	Arg	Arg	His	Pro	Lys	Lys	Leu	Thr	Pro	Leu	Ala	Tyr	Lys	
		35					40					45				

25

CAG	TTT	ATT	CCC	AAT	GTG	GCA	GAG	AAG	ACC	CTA	GGG	GCC	AGT	GGA	AGA	192
Gln	Phe	Ile	Pro	Asn	Val	Ala	Glu	Lys	Thr	Leu	Gly	Ala	Ser	Gly	Arg	
	50					55					60					

30

TAT	GAA	GGG	AAG	ATC	ACA	AGA	AAC	TCC	GAG	AGA	TTT	AAA	GAA	CTA	ACC	240
Tyr	Glu	Gly	Lys	Ile	Thr	Arg	Asn	Ser	Glu	Arg	Phe	Lys	Glu	Leu	Thr	
	65				70				75					80		

CCA	AAT	TAC	AAC	CCT	GAC	ATT	ATT	TTT	AAG	GAT	GAA	GAG	AAC	ACG	GGA	288
Pro	Asn	Tyr	Asn	Pro	Asp	Ile	Ile	Phe	Lys	Asp	Glu	Glu	Asn	Thr	Gly	
			85						90					95		

35

GCT	GAC	AGA	CTG	ATG	ACT	CAG	CGC	TGC	AAG	GAC	AAG	CTG	AAT	GCC	CTG	336
Ala	Asp	Arg	Leu	Met	Thr	Gln	Arg	Cys	Lys	Asp	Lys	Leu	Asn	Ala	Leu	
			100					105					110			

40

GCG	ATC	TCG	GTG	ATG	AAC	CAG	TGG	CCC	GGG	GTG	AAG	CTG	CGG	GTG	ACC	384
Ala	Ile	Ser	Val	Met	Asn	Gln	Trp	Pro	Gly	Val	Lys	Leu	Arg	Val	Thr	
		115				120						125				

45

GAG	GGC	TGG	GAC	GAG	GAT	GGC	CAT	CAC	TCC	GAG	GAA	TCG	CTG	CAC	TAC	432
Glu	Gly	Trp	Asp	Glu	Asp	Gly	His	His	Ser	Glu	Glu	Ser	Leu	His	Tyr	
	130					135					140					

50

GAG	GGT	CGC	GCC	GTG	GAC	ATC	ACC	ACG	TCG	GAT	CGG	GAC	CGC	AGC	AAG	480
Glu	Gly	Arg	Ala	Val	Asp	Ile	Thr	Thr	Ser	Asp	Arg	Asp	Arg	Ser	Lys	
	145				150				155					160		

TAC	GGA	ATG	CTG	GCC	CGC	CTC	GCC	GTC	GAG	GCC	GGC	TTC	GAC	TGG	GTC	528
Tyr	Gly	Met	Leu	Ala	Arg	Leu	Ala	Val	Glu	Ala	Gly	Phe	Asp	Trp	Val	
			165					170						175		

55

TAC	TAC	GAG	TCC	AAG	GCG	CAC	ATC	CAC	TGC	TCC	GTC	AAA	GCA	GAA	AAC	576
Tyr	Tyr	Glu	Ser	Lys	Ala	His	Ile	Cys	Ser	Val	Lys	Ala	Glu	Asn		
			180					185					190			

		TCA	GTG	GCA	GCG	AAA	TCA	GGA	GGC	TGC	¹³⁴ TTC	CCT	GGC	TCA	GCC	ACA	GTG	624
		Ser	Val	Ala	Ala	Lys	Ser	Gly	Gly	Cys	Phe	Pro	Gly	Ser	Ala	Thr	Val	
				195					200						205			
5		CAC	CTG	GAG	CAT	GGA	GGC	ACC	AAG	CTG	GTG	AAG	GAC	CTG	AGC	CCT	GGG	672
		His	Leu	Glu	His	Gly	Gly	Thr	Lys	Leu	Val	Lys	Asp	Leu	Ser	Pro	Gly	
			210					215					220					
10		GAC	CGC	GTG	CTG	GCT	GCT	GAC	GCG	GAC	GGC	CGG	CTG	CTC	TAC	AGT	GAC	720
		Asp	Arg	Val	Leu	Ala	Ala	Asp	Ala	Asp	Gly	Arg	Leu	Leu	Tyr	Ser	Asp	
			225				230					235					240	
15		TTC	CTC	ACC	TTC	CTC	GAC	CGG	ATG	GAC	AGC	TCC	CGA	AAG	CTC	TTC	TAC	768
		Phe	Leu	Thr	Phe	Leu	Asp	Arg	Met	Asp	Ser	Ser	Arg	Lys	Leu	Phe	Tyr	
						245					250					255		
20		GTC	ATC	GAG	ACG	CGG	CAG	CCC	CGG	GCC	CGG	CTG	CTA	CTG	ACG	GCG	GCC	816
		Val	Ile	Glu	Thr	Arg	Gln	Pro	Arg	Ala	Arg	Leu	Leu	Leu	Thr	Ala	Ala	
					260					265					270			
		CAC	CTG	CTC	TTT	GTG	GCC	CCC	CAG	CAC	AAC	CAG	TCG	GAG	GCC	ACA	GGG	864
		His	Leu	Leu	Phe	Val	Ala	Pro	Gln	His	Asn	Gln	Ser	Glu	Ala	Thr	Gly	
				275					280					285				
25		TCC	ACC	AGT	GGC	CAG	GCG	CTC	TTC	GCC	AGC	AAC	GTG	AAG	CCT	GGC	CAA	912
		Ser	Thr	Ser	Gly	Gln	Ala	Leu	Phe	Ala	Ser	Asn	Val	Lys	Pro	Gly	Gln	
			290					295					300					
30		CGT	GTC	TAT	GTG	CTG	GGC	GAG	GGC	GGG	CAG	CAG	CTG	CTG	CCG	GCG	TCT	960
		Arg	Val	Tyr	Val	Leu	Gly	Glu	Gly	Gly	Gln	Gln	Leu	Leu	Pro	Ala	Ser	
			305				310					315					320	
35		GTC	CAC	AGC	GTC	TCA	TTG	CGG	GAG	GAG	GCG	TCC	GGA	GCC	TAC	GCC	CCA	1008
		Val	His	Ser	Val	Ser	Leu	Arg	Glu	Glu	Ala	Ser	Gly	Ala	Tyr	Ala	Pro	
						325					330				335			
40		CTC	ACC	GCC	CAG	GGC	ACC	ATC	CTC	ATC	AAC	CGG	GTG	TTG	GCC	TCC	TGC	1056
		Leu	Thr	Ala	Gln	Gly	Thr	Ile	Leu	Ile	Asn	Arg	Val	Leu	Ala	Ser	Cys	
					340					345					350			
		TAC	GCC	GTC	ATC	GAG	GAG	CAC	AGT	TGG	GCC	CAT	TGG	GCC	TTC	GCA	CCA	1104
		Tyr	Ala	Val	Ile	Glu	Glu	His	Ser	Trp	Ala	His	Trp	Ala	Phe	Ala	Pro	
				355					360					365				
45		TTC	CGC	TTG	GCT	CAG	GGG	CTG	CTG	GCC	GCC	CTC	TGC	CCA	GAT	GGG	GCC	1152
		Phe	Arg	Leu	Ala	Gln	Gly	Leu	Leu	Ala	Ala	Leu	Cys	Pro	Asp	Gly	Ala	
			370					375					380					
50		ATC	CCT	ACT	GCC	GCC	ACC	ACC	ACC	ACT	GGC	ATC	CAT	TGG	TAC	TCA	CGG	1200
		Ile	Pro	Thr	Ala	Ala	Thr	Thr	Thr	Thr	Gly	Ile	His	Trp	Tyr	Ser	Arg	
			385				390				395						400	
55		CTC	CTC	TAC	CGC	ATC	GGC	AGC	TGG	GTG	CTG	GAT	GGT	GAC	GCG	CTG	CAT	1248
		Leu	Leu	Tyr	Arg	Ile	Gly	Ser	Trp	Val	Leu	Asp	Gly	Asp	Ala	Leu	His	
					405						410					415		

CCG CTG GGC ATG GTG GCA CCG GCC AGC TG¹³⁵
 Pro Leu Gly Met Val Ala Pro Ala Ser
 420 425

1277

5

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

10

- (A) LENGTH: 1190 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: both
 (D) TOPOLOGY: linear

15

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

20

- (A) NAME/KEY: CDS
 (B) LOCATION: 1..1191

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

25	ATG GCT CTG CCG GCC AGT CTG TTG CCC CTG TGC TGC TTG GCA CTC TTG Met Ala Leu Pro Ala Ser Leu Leu Pro Leu Cys Cys Leu Ala Leu Leu	48
	1 5 10 15	
30	GCA CTA TCT GCC CAG AGC TGC GGG CCG GGC CGA GGA CCG GTT GGC CGG Ala Leu Ser Ala Gln Ser Cys Gly Pro Gly Arg Gly Pro Val Gly Arg	96
	20 25 30	
35	CGG CGT TAT GTG CGC AAG CAA CTT GTG CCT CTG CTA TAC AAG CAG TTT Arg Arg Tyr Val Arg Lys Gln Leu Val Pro Leu Leu Tyr Lys Gln Phe	144
	35 40 45	
40	GTG CCC AGT ATG CCC GAG CGG ACC CTG GGC GCG AGT GGG CCA GCG GAG Val Pro Ser Met Pro Glu Arg Thr Leu Gly Ala Ser Gly Pro Ala Glu	192
	50 55 60	
45	GGG AGG GTA ACA AGG GGG TCG GAG CGC TTC CGG GAC CTC GTA CCC AAC Gly Arg Val Thr Arg Gly Ser Glu Arg Phe Arg Asp Leu Val Pro Asn	240
	65 70 75 80	
50	TAC AAC CCC GAC ATA ATC TTC AAG GAT GAG GAG AAC AGC GGC GCA GAC Tyr Asn Pro Asp Ile Ile Phe Lys Asp Glu Glu Asn Ser Gly Ala Asp	288
	85 90 95	
55	CGC CTG ATG ACA GAG CGT TGC AAA GAG CGG GTG AAC GCT CTA GCC ATC Arg Leu Met Thr Glu Arg Cys Lys Glu Arg Val Asn Ala Leu Ala Ile	336
	100 105 110	
60	GCG GTG ATG AAC ATG TGG CCC GGA GTA CGC CTA CGT GTG ACT GAA GGC Ala Val Met Asn Met Trp Pro Gly Val Arg Leu Arg Val Thr Glu Gly	384
	115 120 125	
65	TGG GAC GAG GAC GGC CAC CAC GCA CAG GAT TCA CTC CAC TAC GAA GGC Trp Asp Glu Asp Gly His His Ala Gln Asp Ser Leu His Tyr Glu Gly	432
	130 135 140	

										136									
	CGT	GCC	TTG	GAC	ATC	ACC	ACG	TCT	GAC	CGT	GAC	CGT	AAT	AAG	TAT	GGT		480	
	Arg	Ala	Leu	Asp	Ile	Thr	Thr	Ser	Asp	Arg	Asp	Arg	Asn	Lys	Tyr	Gly			
	145					150					155					160			
5	TTG	TTG	GCG	CGC	CTA	GCT	GTG	GAA	GCC	GGA	TTC	GAC	TGG	GTC	TAC	TAC		528	
	Leu	Leu	Ala	Arg	Leu	Ala	Val	Glu	Ala	Gly	Phe	Asp	Trp	Val	Tyr	Tyr			
					165					170					175				
10	GAG	TCC	CGC	AAC	CAC	ATC	CAC	GTA	TCG	GTC	AAA	GCT	GAT	AAC	TCA	CTG		576	
	Glu	Ser	Arg	Asn	His	Ile	His	Val	Ser	Val	Lys	Ala	Asp	Asn	Ser	Leu			
				180					185					190					
15	GCG	GTC	CGA	GCC	GGA	GGC	TGC	TTT	CCG	GGA	AAT	GCC	ACG	GTG	CGC	TTG		624	
	Ala	Val	Arg	Ala	Gly	Gly	Cys	Phe	Pro	Gly	Asn	Ala	Thr	Val	Arg	Leu			
			195					200					205						
20	CGG	AGC	GGC	GAA	CGG	AAG	GGG	CTG	AGG	GAA	CTA	CAT	CGT	GGT	GAC	TGG		672	
	Arg	Ser	Gly	Glu	Arg	Lys	Gly	Leu	Arg	Glu	Leu	His	Arg	Gly	Asp	Trp			
		210					215					220							
	GTA	CTG	GCC	GCT	GAT	GCA	GCG	GGC	CGA	GTG	GTA	CCC	ACG	CCA	GTG	CTG		720	
	Val	Leu	Ala	Ala	Asp	Ala	Ala	Gly	Arg	Val	Val	Pro	Thr	Pro	Val	Leu			
	225					230				235					240				
25	CTC	TTC	CTG	GAC	CGG	GAT	CTG	CAG	CGC	CGC	GCC	TCG	TTC	GTG	GCT	GTG		768	
	Leu	Phe	Leu	Asp	Arg	Asp	Leu	Gln	Arg	Arg	Ala	Ser	Phe	Val	Ala	Val			
					245				250						255				
30	GAG	ACC	GAG	CGG	CCT	CCG	CGC	AAA	CTG	TTG	CTC	ACA	CCC	TGG	CAT	CTG		816	
	Glu	Thr	Glu	Arg	Pro	Pro	Arg	Lys	Leu	Leu	Leu	Thr	Pro	Trp	His	Leu			
				260				265						270					
35	GTG	TTC	GCT	GCT	CGC	GGG	CCA	GCG	CCT	GCT	CCA	GGT	GAC	TTT	GCA	CCG		864	
	Val	Phe	Ala	Ala	Arg	Gly	Pro	Ala	Pro	Ala	Pro	Gly	Asp	Phe	Ala	Pro			
			275				280						285						
40	GTG	TTC	GCG	CGC	CGC	TTA	CGT	GCT	GGC	GAC	TCG	GTG	CTG	GCT	CCC	GGC		912	
	Val	Phe	Ala	Arg	Arg	Leu	Arg	Ala	Gly	Asp	Ser	Val	Leu	Ala	Pro	Gly			
		290				295					300								
	GGG	GAC	GCG	CTC	CAG	CCG	GCG	CGC	GTA	GCC	CGC	GTG	GCG	CGC	GAG	GAA		960	
	Gly	Asp	Ala	Leu	Gln	Pro	Ala	Arg	Val	Ala	Arg	Val	Ala	Arg	Glu	Glu			
	305				310					315					320				
45	GCC	GTG	GGC	GTG	TTC	GCA	CCG	CTC	ACT	GCG	CAC	GGG	ACG	CTG	CTG	GTC		1008	
	Ala	Val	Gly	Val	Phe	Ala	Pro	Leu	Thr	Ala	His	Gly	Thr	Leu	Leu	Val			
					325				330					335					
50	AAC	GAC	GTC	CTC	GCC	TCC	TGC	TAC	GCG	GTT	CTA	GAG	AGT	CAC	CAG	TGG		1056	
	Asn	Asp	Val	Leu	Ala	Ser	Cys	Tyr	Ala	Val	Leu	Glu	Ser	His	Gln	Trp			
				340				345						350					
55	GCC	CAC	CGC	GCC	TTC	GCC	CCT	TTG	CGG	CTG	CTG	CAC	GCG	CTC	GGG	GCT		1104	
	Ala	His	Arg	Ala	Phe	Ala	Pro	Leu	Arg	Leu	Leu	His	Ala	Leu	Gly	Ala			
			355				360						365						

137

CTG CTC CCT GGG GGT GCA GTC CAG CCG ACT GGC ATG CAT TGG TAC TCT	1152
Leu Leu Pro Gly Gly Ala Val Gln Pro Thr Gly Met His Trp Tyr Ser	
370 375 380	

5	CGC CTC CTT TAC CGC TTG GCC GAG GAG TTA ATG GGC TG	1190
	Arg Leu Leu Tyr Arg Leu Ala Glu Glu Leu Met Gly	
	385 390 395	

10 (2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- 15 (A) LENGTH: 1056 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: both
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

20 (ix) FEATURE:

- (A) NAME/KEY: CDS
 (B) LOCATION: 1..1056

25 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

30	GAG CGC TTC AAA GAG CTC ACC CCC AAC TAC AAT CCC GAC ATC ATC TTC	48
	Glu Arg Phe Lys Glu Leu Thr Pro Asn Tyr Asn Pro Asp Ile Ile Phe	
	1 5 10 15	
35	AAG GAC GAG GAG AAC ACG GGT GCC GAC CGC CTC ATG ACC CAG CGC TGC	96
	Lys Asp Glu Glu Asn Thr Gly Ala Asp Arg Leu Met Thr Gln Arg Cys	
	20 25 30	
40	AAG GAC CGT CTG AAC TCA CTG GCC ATC TCT GTC ATG AAC CAG TGG CCT	144
	Lys Asp Arg Leu Asn Ser Leu Ala Ile Ser Val Met Asn Gln Trp Pro	
	35 40 45	
45	GGT GTG AAA CTG CGG GTG ACC GAA GGC CGG GAT GAA GAT GGC CAT CAC	192
	Gly Val Lys Leu Arg Val Thr Glu Gly Arg Asp Glu Asp Gly His His	
	50 55 60	
50	TCA GAG GAG TCT TTA CAC TAT GAG GGC CGC GCG GTG GAT ATC ACC ACC	240
	Ser Glu Glu Ser Leu His Tyr Glu Gly Arg Ala Val Asp Ile Thr Thr	
	65 70 75 80	
55	TCA GAC CGT GAC CGA AAT AAG TAT GGA CTG CTG GCG CGC TTA GCA GTG	288
	Ser Asp Arg Asp Arg Asn Lys Tyr Gly Leu Leu Ala Arg Leu Ala Val	
	85 90 95	
60	GAG GCC GGC TTC GAC TGG GTG TAT TAC GAG TCC AAG GCC CAC GTG CAT	336
	Glu Ala Gly Phe Asp Trp Val Tyr Tyr Glu Ser Lys Ala His Val His	
	100 105 110	
65	TGC TCT GTC AAG TCT GAG CAT TCG GCC GCT GCC AAG ACA GGT GGC TGC	384
	Cys Ser Val Lys Ser Glu His Ser Ala Ala Ala Lys Thr Gly Gly Cys	
	115 120 125	

	TTT CCT GCC GGA GCC CAG GTG CGC CTA	138	GAG AAC GGG GAG CGT GTG GCC	432
	Phe Pro Ala Gly Ala Gln Val Arg Leu Glu Asn Gly Glu Arg Val Ala	130 135 140		
5	CTG TCA GCT GTA AAG CCA GGA GAC CGG GTG CTG GCC ATG GGG GAG GAT		480	
	Leu Ser Ala Val Lys Pro Gly Asp Arg Val Leu Ala Met Gly Glu Asp	145 150 155 160		
10	GGG ACC CCC ACC TTC AGT GAT GTG CTT ATT TTC CTG GAC CGC GAG CCA		528	
	Gly Thr Pro Thr Phe Ser Asp Val Leu Ile Phe Leu Asp Arg Glu Pro	165 170 175		
15	AAC CGG CTG AGA GCT TTC CAG GTC ATC GAG ACT CAG GAT CCT CCG CGT		576	
	Asn Arg Leu Arg Ala Phe Gln Val Ile Glu Thr Gln Asp Pro Pro Arg	180 185 190		
20	CGG CTG GCG CTC ACG CCT GCC CAC CTG CTC TTC ATT GCG GAC AAT CAT		624	
	Arg Leu Ala Leu Thr Pro Ala His Leu Leu Phe Ile Ala Asp Asn His	195 200 205		
25	ACA GAA CCA GCA GCC CAC TTC CGG GCC ACA TTT GCC AGC CAT GTG CAA		672	
	Thr Glu Pro Ala Ala His Phe Arg Ala Thr Phe Ala Ser His Val Gln	210 215 220		
30	CCA GGC CAA TAT GTG CTG GTA TCA GGG GTA CCA GGC CTC CAG CCT GCT		720	
	Pro Gly Gln Tyr Val Leu Val Ser Gly Val Pro Gly Leu Gln Pro Ala	225 230 235 240		
35	CGG GTG GCA GCT GTC TCC ACC CAC GTG GCC CTT GGG TCC TAT GCT CCT		768	
	Arg Val Ala Ala Val Ser Thr His Val Ala Leu Gly Ser Tyr Ala Pro	245 250 255		
40	CTC ACA AGG CAT GGG ACA CTT GTG GTG GAG GAT GTG GTG GCC TCC TGC		816	
	Leu Thr Arg His Gly Thr Leu Val Val Glu Asp Val Val Ala Ser Cys	260 265 270		
45	TTT GCA GCT GTG GCT GAC CAC CAT CTG GCT CAG TTG GCC TTC TGG CCC		864	
	Phe Ala Ala Val Ala Asp His His Leu Ala Gln Leu Ala Phe Trp Pro	275 280 285		
50	CTG CGA CTG TTT CCC AGT TTG GCA TGG GGC AGC TGG ACC CCA AGT GAG		912	
	Leu Arg Leu Phe Pro Ser Leu Ala Trp Gly Ser Trp Thr Pro Ser Glu	290 295 300		
55	GGT GTT CAC TCC TAC CCT CAG ATG CTC TAC CGC CTG GGG CGT CTC TTG		960	
	Gly Val His Ser Tyr Pro Gln Met Leu Tyr Arg Leu Gly Arg Leu Leu	305 310 315 320		
60	CTA GAA GAG AGC ACC TTC CAT CCA CTG GGC ATG TCT GGG GCA GGA AGC		1008	
	Leu Glu Glu Ser Thr Phe His Pro Leu Gly Met Ser Gly Ala Gly Ser	325 330 335		
65	TGAAGGGACT CTAACCACTG CCCTCCTGGA ACTGCTGTGC GTGGATCC		1056	

(2) INFORMATION FOR SEQ ID NO:4:

139

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1313 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: both
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

- (A) NAME/KEY: CDS
 (B) LOCATION: 1..1314

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

20	ATG CTG CTG CTG CTG GCC AGA TGT TTT CTG GTG ATC CTT GCT TCC TCG	48
	Met Leu Leu Leu Leu Ala Arg Cys Phe Leu Val Ile Leu Ala Ser Ser	
	1 5 10 15	
25	CTG CTG GTG TGC CCC GGG CTG GCC TGT GGG CCC GGC AGG GGG TTT GGA	96
	Leu Leu Val Cys Pro Gly Leu Ala Cys Gly Pro Gly Arg Gly Phe Gly	
	20 25 30	
30	AAG AGG CGG CAC CCC AAA AAG CTG ACC CCT TTA GCC TAC AAG CAG TTT	144
	Lys Arg Arg His Pro Lys Lys Leu Thr Pro Leu Ala Tyr Lys Gln Phe	
	35 40 45	
35	ATT CCC AAC GTA GCC GAG AAG ACC CTA GGG GCC AGC GGC AGA TAT GAA	192
	Ile Pro Asn Val Ala Glu Lys Thr Leu Gly Ala Ser Gly Arg Tyr Glu	
	50 55 60	
40	GGG AAG ATC ACA AGA AAC TCC GAA CGA TTT AAG GAA CTC ACC CCC AAT	240
	Gly Lys Ile Thr Arg Asn Ser Glu Arg Phe Lys Glu Leu Thr Pro Asn	
	65 70 75 80	
45	TAC AAC CCC GAC ATC ATA TTT AAG GAT GAG GAA AAC ACG GGA GCA GAC	288
	Tyr Asn Pro Asp Ile Ile Phe Lys Asp Glu Glu Asn Thr Gly Ala Asp	
	85 90 95	
50	CGG CTG ATG ACT CAG AGG TGC AAA GAC AAG TTA AAT GCC TTG GCC ATC	336
	Arg Leu Met Thr Gln Arg Cys Lys Asp Lys Leu Asn Ala Leu Ala Ile	
	100 105 110	
55	TCT GTG ATG AAC CAG TGG CCT GGA GTG AGG CTG CGA GTG ACC GAG GGC	384
	Ser Val Met Asn Gln Trp Pro Gly Val Arg Leu Arg Val Thr Glu Gly	
	115 120 125	
60	TGG GAT GAG GAC GGC CAT CAT TCA GAG GAG TCT CTA CAC TAT GAG GGT	432
	Trp Asp Glu Asp Gly His His Ser Glu Glu Ser Leu His Tyr Glu Gly	
	130 135 140	
65	CGA GCA GTG GAC ATC ACC ACG TCC GAC CGG GAC CGC AGC AAG TAC GGC	480
	Arg Ala Val Asp Ile Thr Ser Asp Arg Asp Arg Ser Lys Tyr Gly	
	145 150 155 160	

	ATG CTG GCT CGC CTG GCT GTG GAA GCA	140	GGT TTC GAC TGG GTC TAC TAT	528
	Met Leu Ala Arg Leu Ala Val Glu Ala	165	Gly Phe Asp Trp Val Tyr Tyr	
			170 175	
5	GAA TCC AAA GCT CAC ATC CAC TGT TCT GTG AAA GCA GAG AAC TCC GTG			576
	Glu Ser Lys Ala His Ile His Cys Ser Val Lys Ala Glu Asn Ser Val	180 185	190	
10	GCG GCC AAA TCC GGC GGC TGT TTC CCG GGA TCC GCC ACC GTG CAC CTG			624
	Ala Ala Lys Ser Gly Gly Cys Phe Pro Gly Ser Ala Thr Val His Leu	195 200	205	
15	GAG CAG GGC GGC ACC AAG CTG GTG AAG GAC TTA CGT CCC GGA GAC CGC			672
	Glu Gln Gly Gly Thr Lys Leu Val Lys Asp Leu Arg Pro Gly Asp Arg	210 215	220	
20	GTG CTG GCG GCT GAC GAC CAG GGC CGG CTG CTG TAC AGC GAC TTC CTC			720
	Val Leu Ala Ala Asp Asp Gln Gly Arg Leu Leu Tyr Ser Asp Phe Leu	225 230	235 240	
	ACC TTC CTG GAC CGC GAC GAA GGC GCC AAG AAG GTC TTC TAC GTG ATC			768
	Thr Phe Leu Asp Arg Asp Glu Gly Ala Lys Lys Val Phe Tyr Val Ile	245 250	255	
25	GAG ACG CTG GAG CCG CGC GAG CGC CTG CTG CTC ACC GCC GCG CAC CTG			816
	Glu Thr Leu Glu Pro Arg Glu Arg Leu Leu Leu Thr Ala Ala His Leu	260 265	270	
30	CTC TTC GTG GCG CCG CAC AAC GAC TCG GGG CCC ACG CCC GGG CCA AGC			864
	Leu Phe Val Ala Pro His Asn Asp Ser Gly Pro Thr Pro Gly Pro Ser	275 280	285	
35	GCG CTC TTT GCC AGC CGC GTG CGC CCC GGG CAG CGC GTG TAC GTG GTG			912
	Ala Leu Phe Ala Ser Arg Val Arg Pro Gly Gln Arg Val Tyr Val Val	290 295	300	
40	GCT GAA CGC GGC GGG GAC CGC CGG CTG CTG CCC GCC GCG GTG CAC AGC			960
	Ala Glu Arg Gly Gly Asp Arg Arg Leu Leu Pro Ala Ala Val His Ser	305 310	315 320	
	GTG ACG CTG CGA GAG GAG GAG GCG GGC GCG TAC GCG CCG CTC ACG GCG			1008
	Val Thr Leu Arg Glu Glu Glu Ala Gly Ala Tyr Ala Pro Leu Thr Ala	325 330	335	
45	CAC GGC ACC ATT CTC ATC AAC CGG GTG CTC GCC TCG TGC TAC GCT GTC			1056
	His Gly Thr Ile Leu Ile Asn Arg Val Leu Ala Ser Cys Tyr Ala Val	340 345	350	
50	ATC GAG GAG CAC AGC TGG GCA CAC CGG GCC TTC GCG CCT TTC CGC CTG			1104
	Ile Glu Glu His Ser Trp Ala His Arg Ala Phe Ala Pro Phe Arg Leu	355 360	365	
55	GCG CAC GCG CTG CTG GCC GCG CTG GCA CCC GCC CGC ACG GAC GGC GGG			1152
	Ala His Ala Leu Leu Ala Ala Leu Ala Pro Ala Arg Thr Asp Gly Gly	370 375	380	

141

	GGC GGG GGC AGC ATC CCT GCA GCG CAA TCT GCA ACG GAA GCG AGG GGC	1200
	Gly Gly Gly Ser Ile Pro Ala Ala Gln Ser Ala Thr Glu Ala Arg Gly	
	385 390 395 400	
5	GCG GAG CCG ACT GCG GGC ATC CAC TGG TAC TCG CAG CTG CTC TAC CAC	1248
	Ala Glu Pro Thr Ala Gly Ile His Trp Tyr Ser Gln Leu Leu Tyr His	
	405 410 415	
10	ATT GGC ACC TGG CTG TTG GAC AGC GAG ACC ATG CAT CCC TTG GGA ATG	1296
	Ile Gly Thr Trp Leu Leu Asp Ser Glu Thr Met His Pro Leu Gly Met	
	420 425 430	
15	GCG GTC AAG TCC AGC TG	1313
	Ala Val Lys Ser Ser	
	435	

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1256 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: both
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 1..1257

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

35	ATG CGG CTT TTG ACG AGA GTG CTG CTG GTG TCT CTT CTC ACT CTG TCC	48
	Met Arg Leu Leu Thr Arg Val Leu Leu Val Ser Leu Leu Thr Leu Ser	
	1 5 10 15	
40	TTG GTG GTG TCC GGA CTG GCC TGC GGT CCT GGC AGA GGC TAC GGC AGA	96
	Leu Val Val Ser Gly Leu Ala Cys Gly Pro Gly Arg Gly Tyr Gly Arg	
	20 25 30	
45	AGA AGA CAT CCG AAG AAG CTG ACA CCT CTC GCC TAC AAG CAG TTC ATA	144
	Arg Arg His Pro Lys Lys Leu Thr Pro Leu Ala Tyr Lys Gln Phe Ile	
	35 40 45	
50	CCT AAT GTC GCG GAG AAG ACC TTA GGG GCC AGC GGC AGA TAC GAG GGC	192
	Pro Asn Val Ala Glu Lys Thr Leu Gly Ala Ser Gly Arg Tyr Glu Gly	
	50 55 60	
55	AAG ATA ACG CGC AAT TCG GAG AGA TTT AAA GAA CTT ACT CCA AAT TAC	240
	Lys Ile Thr Arg Asn Ser Glu Arg Phe Lys Glu Leu Thr Pro Asn Tyr	
	65 70 75 80	
	AAT CCC GAC ATT ATC TTT AAG GAT GAG GAG AAC ACG GGA GCG GAC AGG	288
	Asn Pro Asp Ile Ile Phe Lys Asp Glu Glu Asn Thr Gly Ala Asp Arg	
	85 90 95	

		CTC	ATG	ACA	CAG	AGA	TGC	AAA	GAC	AAG	CTG	AAC	TCG	CTG	GCC	ATC	TCT	336
		Leu	Met	Thr	Gln	Arg	Cys	Lys	Asp	Lys	Leu	Asn	Ser	Leu	Ala	Ile	Ser	
					100					105					110			
5		GTA	ATG	AAC	CAC	TGG	CCA	GGG	GTT	AAG	CTG	CGT	GTG	ACA	GAG	GGC	TGG	384
		Val	Met	Asn	His	Trp	Pro	Gly	Val	Lys	Leu	Arg	Val	Thr	Glu	Gly	Trp	
				115					120					125				
10		GAT	GAG	GAC	GGT	CAC	CAT	TTT	GAA	GAA	TCA	CTC	CAC	TAC	GAG	GGA	AGA	432
		Asp	Glu	Asp	Gly	His	His	Phe	Glu	Glu	Ser	Leu	His	Tyr	Glu	Gly	Arg	
			130					135					140					
15		GCT	GTT	GAT	ATT	ACC	ACC	TCT	GAC	CGA	GAC	AAG	AGC	AAA	TAC	GGG	ACA	480
		Ala	Val	Asp	Ile	Thr	Thr	Ser	Asp	Arg	Asp	Lys	Ser	Lys	Tyr	Gly	Thr	
		145				150						155					160	
20		CTG	TCT	CGC	CTA	GCT	GTG	GAG	GCT	GGA	TTT	GAC	TGG	GTC	TAT	TAC	GAG	528
		Leu	Ser	Arg	Leu	Ala	Val	Glu	Ala	Gly	Phe	Asp	Trp	Val	Tyr	Tyr	Glu	
					165						170					175		
25		TCC	AAA	GCC	CAC	ATT	CAT	TGC	TCT	GTC	AAA	GCA	GAA	AAT	TCG	GTT	GCT	576
		Ser	Lys	Ala	His	Ile	His	Cys	Ser	Val	Lys	Ala	Glu	Asn	Ser	Val	Ala	
				180						185					190			
30		GCG	AAA	TCT	GGG	GGC	TGT	TTC	CCA	GGT	TCG	GCT	CTG	GTC	TCG	CTC	CAG	624
		Ala	Lys	Ser	Gly	Gly	Cys	Phe	Pro	Gly	Ser	Ala	Leu	Val	Ser	Leu	Gln	
			195					200					205					
35		GAC	GGA	GGA	CAG	AAG	GCC	GTG	AAG	GAC	CTG	AAC	CCC	GGA	GAC	AAG	GTG	672
		Asp	Gly	Gly	Gln	Lys	Ala	Val	Lys	Asp	Leu	Asn	Pro	Gly	Asp	Lys	Val	
			210				215						220					
40		CTG	GCG	GCA	GAC	AGC	GCG	GGA	AAC	CTG	GTG	TTC	AGC	GAC	TTC	ATC	ATG	720
		Leu	Ala	Ala	Asp	Ser	Ala	Gly	Asn	Leu	Val	Phe	Ser	Asp	Phe	Ile	Met	
		225				230						235					240	
45		TTC	ACA	GAC	CGA	GAC	TCC	ACG	ACG	CGA	CGT	GTG	TTT	TAC	GTC	ATA	GAA	768
		Phe	Thr	Asp	Arg	Asp	Ser	Thr	Thr	Arg	Arg	Val	Phe	Tyr	Val	Ile	Glu	
					245						250					255		
50		ACG	CAA	GAA	CCC	GTT	GAA	AAG	ATC	ACC	CTC	ACC	GCC	GCT	CAC	CTC	CTT	816
		Thr	Gln	Glu	Pro	Val	Glu	Lys	Ile	Thr	Leu	Thr	Ala	Ala	His	Leu	Leu	
				260					265					270				
55		TTT	GTC	CTC	GAC	AAC	TCA	ACG	GAA	GAT	CTC	CAC	ACC	ATG	ACC	GCC	GCG	864
		Phe	Val	Leu	Asp	Asn	Ser	Thr	Glu	Asp	Leu	His	Thr	Met	Thr	Ala	Ala	
			275					280					285					
60		TAT	GCC	AGC	AGT	GTC	AGA	GCC	GGA	CAA	AAG	GTG	ATG	GTT	GTT	GAT	GAT	912
		Tyr	Ala	Ser	Ser	Val	Arg	Ala	Gly	Gln	Lys	Val	Met	Val	Val	Asp	Asp	
			290					295					300					
65		AGC	GGT	CAG	CTT	AAA	TCT	GTC	ATC	GTG	CAG	CGG	ATA	TAC	ACG	GAG	GAG	960
		Ser	Gly	Gln	Leu	Lys	Ser	Val	Ile	Val	Gln	Arg	Ile	Tyr	Thr	Glu	Glu	
		305				310					315						320	

	CAG CGG GGC TCG TTC GCA CCA GTG ACT GCA CAT GGG ACC ATT GTG GTC	1008
	Gln Arg Gly Ser Phe Ala Pro Val Thr Ala His Gly Thr Ile Val Val	
	325 330 335	
5	GAC AGA ATA CTG GCG TCC TGT TAC GCC GTA ATA GAG GAC CAG GGG CTT	1056
	Asp Arg Ile Leu Ala Ser Cys Tyr Ala Val Ile Glu Asp Gln Gly Leu	
	340 345 350	
10	GCG CAT TTG GCC TTC GCG CCC GCC AGG CTC TAT TAT TAC GTG TCA TCA	1104
	Ala His Leu Ala Phe Ala Pro Ala Arg Leu Tyr Tyr Tyr Val Ser Ser	
	355 360 365	
15	TTC CTG TCC CCC AAA ACT CCA GCA GTC GGT CCA ATG CGA CTT TAC AAC	1152
	Phe Leu Ser Pro Lys Thr Pro Ala Val Gly Pro Met Arg Leu Tyr Asn	
	370 375 380	
20	AGG AGG GGG TCC ACT GGT ACT CCA GGC TCC TGT CAT CAA ATG GGA ACG	1200
	Arg Arg Gly Ser Thr Gly Thr Pro Gly Ser Cys His Gln Met Gly Thr	
	385 390 395 400	
	TGG CTT TTG GAC AGC AAC ATG CTT CAT CCT TTG GGG ATG TCA GTA AAC	1248
	Trp Leu Leu Asp Ser Asn Met Leu His Pro Leu Gly Met Ser Val Asn	
	405 410 415	
25	TCA AGC TG	1256
	Ser Ser	
30	(2) INFORMATION FOR SEQ ID NO:6:	
	(i) SEQUENCE CHARACTERISTICS:	
	(A) LENGTH: 1425 base pairs	
	(B) TYPE: nucleic acid	
35	(C) STRANDEDNESS: single	
	(D) TOPOLOGY: linear	
	(ii) MOLECULE TYPE: cDNA	
40	(ix) FEATURE:	
	(A) NAME/KEY: CDS	
	(B) LOCATION: 1..1425	
45	(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:	
	ATG CTG CTG CTG GCG AGA TGT CTG CTG CTA GTC CTC GTC TCC TCG CTG	48
50	Met Leu Leu Leu Ala Arg Cys Leu Leu Leu Val Leu Val Ser Ser Leu	
	1 5 10 15	
	CTG GTA TGC TCG GGA CTG GCG TGC GGA CCG GGC AGG GGG TTC GGG AAG	96
	Leu Val Cys Ser Gly Leu Ala Cys Gly Pro Gly Arg Gly Phe Gly Lys	
	20 25 30	
55	AGG AGG CAC CCC AAA AAG CTG ACC CCT TTA GCC TAC AAG CAG TTT ATC	144
	Arg Arg His Pro Lys Lys Leu Thr Pro Leu Ala Tyr Lys Gln Phe Ile	
	35 40 45	

	CCC	AAT	GTG	GCC	GAG	AAG	ACC	CTA	GGC	¹⁴⁴ GCC	AGC	GGA	AGG	TAT	GAA	GGG	192
	Pro	Asn	Val	Ala	Glu	Lys	Thr	Leu	Gly	Ala	Ser	Gly	Arg	Tyr	Glu	Gly	
	50						55					60					
5	AAG	ATC	TCC	AGA	AAC	TCC	GAG	CGA	TTT	AAG	GAA	CTC	ACC	CCC	AAT	TAC	240
	Lys	Ile	Ser	Arg	Asn	Ser	Glu	Arg	Phe	Lys	Glu	Leu	Thr	Pro	Asn	Tyr	
	65					70					75					80	
10	AAC	CCC	GAC	ATC	ATA	TTT	AAG	GAT	GAA	GAA	AAC	ACC	GGA	GCG	GAC	AGG	288
	Asn	Pro	Asp	Ile	Ile	Phe	Lys	Asp	Glu	Glu	Asn	Thr	Gly	Ala	Asp	Arg	
					85					90					95		
15	CTG	ATG	ACT	CAG	AGG	TGT	AAG	GAC	AAG	TTG	AAC	GCT	TTG	GCC	ATC	TCG	336
	Leu	Met	Thr	Gln	Arg	Cys	Lys	Asp	Lys	Leu	Asn	Ala	Leu	Ala	Ile	Ser	
				100					105					110			
20	GTG	ATG	AAC	CAG	TGG	CCA	GGA	GTG	AAA	CTG	CGG	GTG	ACC	GAG	GGC	TGG	384
	Val	Met	Asn	Gln	Trp	Pro	Gly	Val	Lys	Leu	Arg	Val	Thr	Glu	Gly	Trp	
			115					120					125				
	GAC	GAA	GAT	GGC	CAC	CAC	TCA	GAG	GAG	TCT	CTG	CAC	TAC	GAG	GGC	CGC	432
	Asp	Glu	Asp	Gly	His	His	Ser	Glu	Glu	Ser	Leu	His	Tyr	Glu	Gly	Arg	
	130						135					140					
25	GCA	GTG	GAC	ATC	ACC	ACG	TCT	GAC	CGC	GAC	CGC	AGC	AAG	TAC	GGC	ATG	480
	Ala	Val	Asp	Ile	Thr	Thr	Ser	Asp	Arg	Asp	Arg	Ser	Lys	Tyr	Gly	Met	
	145					150					155					160	
30	CTG	GCC	CGC	CTG	GCG	GTG	GAG	GCC	GGC	TTC	GAC	TGG	GTG	TAC	TAC	GAG	528
	Leu	Ala	Arg	Leu	Ala	Val	Glu	Ala	Gly	Phe	Asp	Trp	Val	Tyr	Tyr	Glu	
				165					170						175		
35	TCC	AAG	GCA	CAT	ATC	CAC	TGC	TCG	GTG	AAA	GCA	GAG	AAC	TCG	GTG	GCG	576
	Ser	Lys	Ala	His	Ile	His	Cys	Ser	Val	Lys	Ala	Glu	Asn	Ser	Val	Ala	
				180					185					190			
40	GCC	AAA	TCG	GGA	GGC	TGC	TTC	CCG	GGC	TCG	GCC	ACG	GTG	CAC	CTG	GAG	624
	Ala	Lys	Ser	Gly	Gly	Cys	Phe	Pro	Gly	Ser	Ala	Thr	Val	His	Leu	Glu	
			195					200					205				
	CAG	GGC	GGC	ACC	AAG	CTG	GTG	AAG	GAC	CTG	AGC	CCC	GGG	GAC	CGC	GTG	672
	Gln	Gly	Gly	Thr	Lys	Leu	Val	Lys	Asp	Leu	Ser	Pro	Gly	Asp	Arg	Val	
	210					215						220					
45	CTG	GCG	GCG	GAC	GAC	CAG	GGC	CGG	CTG	CTC	TAC	AGC	GAC	TTC	CTC	ACT	720
	Leu	Ala	Ala	Asp	Asp	Gln	Gly	Arg	Leu	Leu	Tyr	Ser	Asp	Phe	Leu	Thr	
	225					230					235					240	
50	TTC	CTG	GAC	CGC	GAC	GAC	GGC	GCC	AAG	AAG	GTC	TTC	TAC	GTG	ATC	GAG	768
	Phe	Leu	Asp	Arg	Asp	Asp	Gly	Ala	Lys	Lys	Val	Phe	Tyr	Val	Ile	Glu	
				245					250						255		
55	ACG	CGG	GAG	CCG	CGC	GAG	CGC	CTG	CTG	CTC	ACC	GCC	GCG	CAC	CTG	CTC	816
	Thr	Arg	Glu	Pro	Arg	Glu	Arg	Leu	Leu	Leu	Thr	Ala	Ala	His	Leu	Leu	
				260				265						270			

	TTT GTG GCG CCG CAC AAC GAC TCG GCC ACC GGG GAG CCC GAG GCG TCC	864
	Phe Val Ala Pro His Asn Asp Ser Ala Thr Gly Glu Pro Glu Ala Ser	
	275 280 285	
5	TCG GGC TCG GGG CCG CCT TCC GGG GGC GCA CTG GGG CCT CGG GCG CTG	912
	Ser Gly Ser Gly Pro Pro Ser Gly Gly Ala Leu Gly Pro Arg Ala Leu	
	290 295 300	
10	TTC GCC AGC CGC GTG CGC CCG GGC CAG CGC GTG TAC GTG GTG GCC GAG	960
	Phe Ala Ser Arg Val Arg Pro Gly Gln Arg Val Tyr Val Val Ala Glu	
	305 310 315 320	
15	CGT GAC GGG GAC CGC CGG CTC CTG CCC GCC GCT GTG CAC AGC GTG ACC	1008
	Arg Asp Gly Asp Arg Arg Leu Leu Pro Ala Ala Val His Ser Val Thr	
	325 330 335	
20	CTA AGC GAG GAG GCC GCG GGC GCC TAC GCG CCG CTC ACG GCC CAG GGC	1056
	Leu Ser Glu Glu Ala Ala Gly Ala Tyr Ala Pro Leu Thr Ala Gln Gly	
	340 345 350	
	ACC ATT CTC ATC AAC CGG GTG CTG GCC TCG TGC TAC GCG GTC ATC GAG	1104
	Thr Ile Leu Ile Asn Arg Val Leu Ala Ser Cys Tyr Ala Val Ile Glu	
	355 360 365	
25	GAG CAC AGC TGG GCG CAC CGG GCC TTC GCG CCC TTC CGC CTG GCG CAC	1152
	Glu His Ser Trp Ala His Arg Ala Phe Ala Pro Phe Arg Leu Ala His	
	370 375 380	
30	GCG CTC CTG GCT GCA CTG GCG CCC GCG CGC ACG GAC CGC GGC GGG GAC	1200
	Ala Leu Leu Ala Ala Leu Ala Pro Ala Arg Thr Asp Arg Gly Gly Asp	
	385 390 395 400	
35	AGC GGC GGC GGG GAC CGC GGG GGC GGC GGC GGC AGA GTA GCC CTA ACC	1248
	Ser Gly Gly Gly Asp Arg Gly Gly Gly Gly Gly Arg Val Ala Leu Thr	
	405 410 415	
40	GCT CCA GGT GCT GCC GAC GCT CCG GGT GCG GGG GCC ACC GCG GGC ATC	1296
	Ala Pro Gly Ala Ala Asp Ala Pro Gly Ala Gly Ala Thr Ala Gly Ile	
	420 425 430	
	CAC TGG TAC TCG CAG CTG CTC TAC CAA ATA GGC ACC TGG CTC CTG GAC	1344
	His Trp Tyr Ser Gln Leu Leu Tyr Gln Ile Gly Thr Trp Leu Leu Asp	
	435 440 445	
45	AGC GAG GCC CTG CAC CCG CTG GGC ATG GCG GTC AAG TCC AGC NNN AGC	1392
	Ser Glu Ala Leu His Pro Leu Gly Met Ala Val Lys Ser Ser Xaa Ser	
	450 455 460	
50	CGG GGG GCC GGG GGA GGG GCG CGG GAG GGG GCC	1425
	Arg Gly Ala Gly Gly Gly Ala Arg Glu Gly Ala	
	465 470 475	

(2) INFORMATION FOR SEQ ID NO:7:

55

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 940 base pairs

(B) TYPE: nucleic acid

146

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION: 1..940

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

15	CGG CGC CTC ATG ACC CAG CGC TGC AAG GAC CGC CTG AAC TCG CTG GCT	48
	Arg Arg Leu Met Thr Gln Arg Cys Lys Asp Arg Leu Asn Ser Leu Ala	
	1 5 10 15	
20	ATC TCG GTG ATG AAC CAG TGG CCC GGT GTG AAG CTG CGG GTG ACC GAG	96
	Ile Ser Val Met Asn Gln Trp Pro Gly Val Lys Leu Arg Val Thr Glu	
	20 25 30	
25	GGC TGG GAC GAG GAC GGC CAC CAC TCA GAG GAG TCC CTG CAT TAT GAG	144
	Gly Trp Asp Glu Asp Gly His His Ser Glu Glu Ser Leu His Tyr Glu	
	35 40 45	
30	GGC CGC GCG GTG GAC ATC ACC ACA TCA GAC CGC GAC CGC AAT AAG TAT	192
	Gly Arg Ala Val Asp Ile Thr Thr Ser Asp Arg Asp Arg Asn Lys Tyr	
	50 55 60	
35	GGA CTG CTG GCG CGC TTG GCA GTG GAG GCC GGC TTT GAC TGG GTG TAT	240
	Gly Leu Leu Ala Arg Leu Ala Val Glu Ala Gly Phe Asp Trp Val Tyr	
	65 70 75 80	
40	TAC GAG TCA AAG GCC CAC GTG CAT TGC TCC GTC AAG TCC GAG CAC TCG	288
	Tyr Glu Ser Lys Ala His Val His Cys Ser Val Lys Ser Glu His Ser	
	85 90 95	
45	GCC GCA GCC AAG ACG GGC GGC TGC TTC CCT GCC GGA GCC CAG GTA CGC	336
	Ala Ala Ala Lys Thr Gly Gly Cys Phe Pro Ala Gly Ala Gln Val Arg	
	100 105 110	
50	CTG GAG AGT GGG GCG CGT GTG GCC TTG TCA GCC GTG AGG CCG GGA GAC	384
	Leu Glu Ser Gly Ala Arg Val Ala Leu Ser Ala Val Arg Pro Gly Asp	
	115 120 125	
55	CGT GTG CTG GCC ATG GGG GAG GAT GGG AGC CCC ACC TTC AGC GAT GTG	432
	Arg Val Leu Ala Met Gly Glu Asp Gly Ser Pro Thr Phe Ser Asp Val	
	130 135 140	
60	CTC ATT TTC CTG GAC CGC GAG CCC CAC AGG CTG AGA GCC TTC CAG GTC	480
	Leu Ile Phe Leu Asp Arg Glu Pro His Arg Leu Arg Ala Phe Gln Val	
	145 150 155 160	
65	ATC GAG ACT CAG GAC CCC CCA CGC CGC CTG GCA CTC ACA CCC GCT CAC	528
	Ile Glu Thr Gln Asp Pro Pro Arg Arg Leu Ala Leu Thr Pro Ala His	
	165 170 175	

147

[illegible]

(2) INFORMATION FOR SEQ ID NO:8:

40 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 425 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

45 (ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

```

50 Met Val Glu Met Leu Leu Leu Thr Arg Ile Leu Leu Val Gly Phe Ile
    1              5              10              15

    Cys Ala Leu Leu Val Ser Ser Gly Leu Thr Cys Gly Pro Gly Arg Gly
          20              25              30

55 Ile Gly Lys Arg Arg His Pro Lys Lys Leu Thr Pro Leu Ala Tyr Lys
    35              40              45

```

143

	Gln	Phe	Ile	Pro	Asn	Val	Ala	Glu	Lys	Thr	Leu	Gly	Ala	Ser	Gly	Arg	
	50						55					60					
5	Tyr	Glu	Gly	Lys	Ile	Thr	Arg	Asn	Ser	Glu	Arg	Phe	Lys	Glu	Leu	Thr	
	65					70					75					80	
	Pro	Asn	Tyr	Asn	Pro	Asp	Ile	Ile	Phe	Lys	Asp	Glu	Glu	Asn	Thr	Gly	
					85					90					95		
10	Ala	Asp	Arg	Leu	Met	Thr	Gln	Arg	Cys	Lys	Asp	Lys	Leu	Asn	Ala	Leu	
				100					105					110			
	Ala	Ile	Ser	Val	Met	Asn	Gln	Trp	Pro	Gly	Val	Lys	Leu	Arg	Val	Thr	
			115					120					125				
15	Glu	Gly	Trp	Asp	Glu	Asp	Gly	His	His	Ser	Glu	Glu	Ser	Leu	His	Tyr	
	130						135					140					
20	Glu	Gly	Arg	Ala	Val	Asp	Ile	Thr	Thr	Ser	Asp	Arg	Asp	Arg	Ser	Lys	
	145					150					155					160	
	Tyr	Gly	Met	Leu	Ala	Arg	Leu	Ala	Val	Glu	Ala	Gly	Phe	Asp	Trp	Val	
				165						170					175		
25	Tyr	Tyr	Glu	Ser	Lys	Ala	His	Ile	His	Cys	Ser	Val	Lys	Ala	Glu	Asn	
				180					185					190			
	Ser	Val	Ala	Ala	Lys	Ser	Gly	Gly	Cys	Phe	Pro	Gly	Ser	Ala	Thr	Val	
			195					200					205				
30	His	Leu	Glu	His	Gly	Gly	Thr	Lys	Leu	Val	Lys	Asp	Leu	Ser	Pro	Gly	
	210						215					220					
35	Asp	Arg	Val	Leu	Ala	Ala	Asp	Ala	Asp	Gly	Arg	Leu	Leu	Tyr	Ser	Asp	
	225				230						235					240	
	Phe	Leu	Thr	Phe	Leu	Asp	Arg	Met	Asp	Ser	Ser	Arg	Lys	Leu	Phe	Tyr	
				245					250						255		
40	Val	Ile	Glu	Thr	Arg	Gln	Pro	Arg	Ala	Arg	Leu	Leu	Leu	Thr	Ala	Ala	
				260					265					270			
	His	Leu	Leu	Phe	Val	Ala	Pro	Gln	His	Asn	Gln	Ser	Glu	Ala	Thr	Gly	
		275						280					285				
45	Ser	Thr	Ser	Gly	Gln	Ala	Leu	Phe	Ala	Ser	Asn	Val	Lys	Pro	Gly	Gln	
	290						295					300					
50	Arg	Val	Tyr	Val	Leu	Gly	Glu	Gly	Gly	Gln	Gln	Leu	Leu	Pro	Ala	Ser	
	305				310						315				320		
	Val	His	Ser	Val	Ser	Leu	Arg	Glu	Glu	Ala	Ser	Gly	Ala	Tyr	Ala	Pro	
				325					330					335			
55	Leu	Thr	Ala	Gln	Gly	Thr	Ile	Leu	Ile	Asn	Arg	Val	Leu	Ala	Ser	Cys	
				340				345						350			

149

Tyr Ala Val Ile Glu Glu His Ser Trp Ala His Trp Ala Phe Ala Pro
 355 360 365
 5 Phe Arg Leu Ala Gln Gly Leu Leu Ala Ala Leu Cys Pro Asp Gly Ala
 370 375 380
 Ile Pro Thr Ala Ala Thr Thr Thr Thr Gly Ile His Trp Tyr Ser Arg
 385 390 395 400
 10 Leu Leu Tyr Arg Ile Gly Ser Trp Val Leu Asp Gly Asp Ala Leu His
 405 410 415
 Pro Leu Gly Met Val Ala Pro Ala Ser
 420 425
 15

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

- 20 (A) LENGTH: 396 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

25

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Met Ala Leu Pro Ala Ser Leu Leu Pro Leu Cys Cys Leu Ala Leu Leu
 1 5 10 15
 30 Ala Leu Ser Ala Gln Ser Cys Gly Pro Gly Arg Gly Pro Val Gly Arg
 20 25 30
 35 Arg Arg Tyr Val Arg Lys Gln Leu Val Pro Leu Leu Tyr Lys Gln Phe
 35 40 45
 Val Pro Ser Met Pro Glu Arg Thr Leu Gly Ala Ser Gly Pro Ala Glu
 50 55 60
 40 Gly Arg Val Thr Arg Gly Ser Glu Arg Phe Arg Asp Leu Val Pro Asn
 65 70 75 80
 Tyr Asn Pro Asp Ile Ile Phe Lys Asp Glu Glu Asn Ser Gly Ala Asp
 85 90 95
 45 Arg Leu Met Thr Glu Arg Cys Lys Glu Arg Val Asn Ala Leu Ala Ile
 100 105 110
 Ala Val Met Asn Met Trp Pro Gly Val Arg Leu Arg Val Thr Glu Gly
 115 120 125
 Trp Asp Glu Asp Gly His His Ala Gln Asp Ser Leu His Tyr Glu Gly
 130 135 140
 55 Arg Ala Leu Asp Ile Thr Thr Ser Asp Arg Asp Arg Asn Lys Tyr Gly
 145 150 155 160

150

Leu Leu Ala Arg Leu Ala Val Glu Ala Gly Phe Asp Trp Val Tyr Tyr
 165 170 175

5 Glu Ser Arg Asn His Ile His Val Ser Val Lys Ala Asp Asn Ser Leu
 180 185 190

Ala Val Arg Ala Gly Gly Cys Phe Pro Gly Asn Ala Thr Val Arg Leu
 195 200 205

10 Arg Ser Gly Glu Arg Lys Gly Leu Arg Glu Leu His Arg Gly Asp Trp
 210 215 220

Val Leu Ala Ala Asp Ala Ala Gly Arg Val Val Pro Thr Pro Val Leu
 225 230 235 240

15 Leu Phe Leu Asp Arg Asp Leu Gln Arg Arg Ala Ser Phe Val Ala Val
 245 250 255

20 Glu Thr Glu Arg Pro Pro Arg Lys Leu Leu Leu Thr Pro Trp His Leu
 260 265 270

Val Phe Ala Ala Arg Gly Pro Ala Pro Ala Pro Gly Asp Phe Ala Pro
 275 280 285

25 Val Phe Ala Arg Arg Leu Arg Ala Gly Asp Ser Val Leu Ala Pro Gly
 290 295 300

Gly Asp Ala Leu Gln Pro Ala Arg Val Ala Arg Val Ala Arg Glu Glu
 305 310 315 320

30 Ala Val Gly Val Phe Ala Pro Leu Thr Ala His Gly Thr Leu Leu Val
 325 330 335

Asn Asp Val Leu Ala Ser Cys Tyr Ala Val Leu Glu Ser His Gln Trp
 340 345 350

Ala His Arg Ala Phe Ala Pro Leu Arg Leu Leu His Ala Leu Gly Ala
 355 360 365

40 Leu Leu Pro Gly Gly Ala Val Gln Pro Thr Gly Met His Trp Tyr Ser
 370 375 380

Arg Leu Leu Tyr Arg Leu Ala Glu Glu Leu Met Gly
 385 390 395

45

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

50 (A) LENGTH: 336 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

55

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

151

	Glu	Arg	Phe	Lys	Glu	Leu	Thr	Pro	Asn	Tyr	Asn	Pro	Asp	Ile	Ile	Phe	1	5	10	15
5	Lys	Asp	Glu	Glu	Asn	Thr	Gly	Ala	Asp	Arg	Leu	Met	Thr	Gln	Arg	Cys	20	25	30	
	Lys	Asp	Arg	Leu	Asn	Ser	Leu	Ala	Ile	Ser	Val	Met	Asn	Gln	Trp	Pro	35	40	45	
10	Gly	Val	Lys	Leu	Arg	Val	Thr	Glu	Gly	Arg	Asp	Glu	Asp	Gly	His	His	50	55	60	
	Ser	Glu	Glu	Ser	Leu	His	Tyr	Glu	Gly	Arg	Ala	Val	Asp	Ile	Thr	Thr	65	70	75	80
15	Ser	Asp	Arg	Asp	Arg	Asn	Lys	Tyr	Gly	Leu	Leu	Ala	Arg	Leu	Ala	Val	85	90	95	
20	Glu	Ala	Gly	Phe	Asp	Trp	Val	Tyr	Tyr	Glu	Ser	Lys	Ala	His	Val	His	100	105	110	
	Cys	Ser	Val	Lys	Ser	Glu	His	Ser	Ala	Ala	Ala	Lys	Thr	Gly	Gly	Cys	115	120	125	
25	Phe	Pro	Ala	Gly	Ala	Gln	Val	Arg	Leu	Glu	Asn	Gly	Glu	Arg	Val	Ala	130	135	140	
	Leu	Ser	Ala	Val	Lys	Pro	Gly	Asp	Arg	Val	Leu	Ala	Met	Gly	Glu	Asp	145	150	155	160
30	Gly	Thr	Pro	Thr	Phe	Ser	Asp	Val	Leu	Ile	Phe	Leu	Asp	Arg	Glu	Pro	165	170	175	
35	Asn	Arg	Leu	Arg	Ala	Phe	Gln	Val	Ile	Glu	Thr	Gln	Asp	Pro	Pro	Arg	180	185	190	
	Arg	Leu	Ala	Leu	Thr	Pro	Ala	His	Leu	Leu	Phe	Ile	Ala	Asp	Asn	His	195	200	205	
40	Thr	Glu	Pro	Ala	Ala	His	Phe	Arg	Ala	Thr	Phe	Ala	Ser	His	Val	Gln	210	215	220	
	Pro	Gly	Gln	Tyr	Val	Leu	Val	Ser	Gly	Val	Pro	Gly	Leu	Gln	Pro	Ala	225	230	235	240
45	Arg	Val	Ala	Ala	Val	Ser	Thr	His	Val	Ala	Leu	Gly	Ser	Tyr	Ala	Pro	245	250	255	
50	Leu	Thr	Arg	His	Gly	Thr	Leu	Val	Val	Glu	Asp	Val	Val	Ala	Ser	Cys	260	265	270	
	Phe	Ala	Ala	Val	Ala	Asp	His	His	Leu	Ala	Gln	Leu	Ala	Phe	Trp	Pro	275	280	285	
55	Leu	Arg	Leu	Phe	Pro	Ser	Leu	Ala	Trp	Gly	Ser	Trp	Thr	Pro	Ser	Glu	290	295	300	

152

Gly Val His Ser Tyr Pro Gln Met Leu Tyr Arg Leu Gly Arg Leu Leu
 305 310 315 320

5 Leu Glu Glu Ser Thr Phe His Pro Leu Gly Met Ser Gly Ala Gly Ser
 325 330 335

(2) INFORMATION FOR SEQ ID NO:11:

- 10 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 437 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

- 15 (ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

20 Met Leu Leu Leu Leu Ala Arg Cys Phe Leu Val Ile Leu Ala Ser Ser
 1 5 10 15

Leu Leu Val Cys Pro Gly Leu Ala Cys Gly Pro Gly Arg Gly Phe Gly
 20 25 30

25 Lys Arg Arg His Pro Lys Lys Leu Thr Pro Leu Ala Tyr Lys Gln Phe
 35 40 45

Ile Pro Asn Val Ala Glu Lys Thr Leu Gly Ala Ser Gly Arg Tyr Glu
 50 55 60

30 Gly Lys Ile Thr Arg Asn Ser Glu Arg Phe Lys Glu Leu Thr Pro Asn
 65 70 75 80

35 Tyr Asn Pro Asp Ile Ile Phe Lys Asp Glu Glu Asn Thr Gly Ala Asp
 85 90 95

Arg Leu Met Thr Gln Arg Cys Lys Asp Lys Leu Asn Ala Leu Ala Ile
 100 105 110

40 Ser Val Met Asn Gln Trp Pro Gly Val Arg Leu Arg Val Thr Glu Gly
 115 120 125

Trp Asp Glu Asp Gly His His Ser Glu Glu Ser Leu His Tyr Glu Gly
 130 135 140

45 Arg Ala Val Asp Ile Thr Thr Ser Asp Arg Asp Arg Ser Lys Tyr Gly
 145 150 155 160

50 Met Leu Ala Arg Leu Ala Val Glu Ala Gly Phe Asp Trp Val Tyr Tyr
 165 170 175

Glu Ser Lys Ala His Ile His Cys Ser Val Lys Ala Glu Asn Ser Val
 180 185 190

55 Ala Ala Lys Ser Gly Gly Cys Phe Pro Gly Ser Ala Thr Val His Leu
 195 200 205

153

Glu Gln Gly Gly Thr Lys Leu Val Lys Asp Leu Arg Pro Gly Asp Arg
 210 215 220

5 Val Leu Ala Ala Asp Asp Gln Gly Arg Leu Leu Tyr Ser Asp Phe Leu
 225 230 235 240

Thr Phe Leu Asp Arg Asp Glu Gly Ala Lys Lys Val Phe Tyr Val Ile
 245 250 255

10 Glu Thr Leu Glu Pro Arg Glu Arg Leu Leu Leu Thr Ala Ala His Leu
 260 265 270

Leu Phe Val Ala Pro His Asn Asp Ser Gly Pro Thr Pro Gly Pro Ser
 275 280 285

15 Ala Leu Phe Ala Ser Arg Val Arg Pro Gly Gln Arg Val Tyr Val Val
 290 295 300

20 Ala Glu Arg Gly Gly Asp Arg Arg Leu Leu Pro Ala Ala Val His Ser
 305 310 315 320

Val Thr Leu Arg Glu Glu Glu Ala Gly Ala Tyr Ala Pro Leu Thr Ala
 325 330 335

25 His Gly Thr Ile Leu Ile Asn Arg Val Leu Ala Ser Cys Tyr Ala Val
 340 345 350

Ile Glu Glu His Ser Trp Ala His Arg Ala Phe Ala Pro Phe Arg Leu
 355 360 365

30 Ala His Ala Leu Leu Ala Ala Leu Ala Pro Ala Arg Thr Asp Gly Gly
 370 375 380

Gly Gly Gly Ser Ile Pro Ala Ala Gln Ser Ala Thr Glu Ala Arg Gly
 385 390 395 400

Ala Glu Pro Thr Ala Gly Ile His Trp Tyr Ser Gln Leu Leu Tyr His
 405 410 415

40 Ile Gly Thr Trp Leu Leu Asp Ser Glu Thr Met His Pro Leu Gly Met
 420 425 430

Ala Val Lys Ser Ser
 435

45

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:

50 (A) LENGTH: 418 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

55

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

154

	Met	Arg	Leu	Leu	Thr	Arg	Val	Leu	Leu	Val	Ser	Leu	Leu	Thr	Leu	Ser	
	1				5					10					15		
5	Leu	Val	Val	Ser	Gly	Leu	Ala	Cys	Gly	Pro	Gly	Arg	Gly	Tyr	Gly	Arg	
				20					25					30			
	Arg	Arg	His	Pro	Lys	Lys	Leu	Thr	Pro	Leu	Ala	Tyr	Lys	Gln	Phe	Ile	
			35					40					45				
10	Pro	Asn	Val	Ala	Glu	Lys	Thr	Leu	Gly	Ala	Ser	Gly	Arg	Tyr	Glu	Gly	
		50					55					60					
	Lys	Ile	Thr	Arg	Asn	Ser	Glu	Arg	Phe	Lys	Glu	Leu	Thr	Pro	Asn	Tyr	
	65					70					75					80	
15	Asn	Pro	Asp	Ile	Ile	Phe	Lys	Asp	Glu	Glu	Asn	Thr	Gly	Ala	Asp	Arg	
					85					90					95		
20	Leu	Met	Thr	Gln	Arg	Cys	Lys	Asp	Lys	Leu	Asn	Ser	Leu	Ala	Ile	Ser	
				100					105					110			
	Val	Met	Asn	His	Trp	Pro	Gly	Val	Lys	Leu	Arg	Val	Thr	Glu	Gly	Trp	
			115				120						125				
25	Asp	Glu	Asp	Gly	His	His	Phe	Glu	Glu	Ser	Leu	His	Tyr	Glu	Gly	Arg	
		130					135					140					
	Ala	Val	Asp	Ile	Thr	Thr	Ser	Asp	Arg	Asp	Lys	Ser	Lys	Tyr	Gly	Thr	
	145					150					155					160	
30	Leu	Ser	Arg	Leu	Ala	Val	Glu	Ala	Gly	Phe	Asp	Trp	Val	Tyr	Tyr	Glu	
					165				170						175		
	Ser	Lys	Ala	His	Ile	His	Cys	Ser	Val	Lys	Ala	Glu	Asn	Ser	Val	Ala	
35				180					185					190			
	Ala	Lys	Ser	Gly	Gly	Cys	Phe	Pro	Gly	Ser	Ala	Leu	Val	Ser	Leu	Gln	
			195					200					205				
40	Asp	Gly	Gly	Gln	Lys	Ala	Val	Lys	Asp	Leu	Asn	Pro	Gly	Asp	Lys	Val	
		210					215					220					
	Leu	Ala	Ala	Asp	Ser	Ala	Gly	Asn	Leu	Val	Phe	Ser	Asp	Phe	Ile	Met	
	225					230					235					240	
45	Phe	Thr	Asp	Arg	Asp	Ser	Thr	Thr	Arg	Arg	Val	Phe	Tyr	Val	Ile	Glu	
					245					250					255		
	Thr	Gln	Glu	Pro	Val	Glu	Lys	Ile	Thr	Leu	Thr	Ala	Ala	His	Leu	Leu	
50				260					265					270			
	Phe	Val	Leu	Asp	Asn	Ser	Thr	Glu	Asp	Leu	His	Thr	Met	Thr	Ala	Ala	
			275					280					285				
55	Tyr	Ala	Ser	Ser	Val	Arg	Ala	Gly	Gln	Lys	Val	Met	Val	Val	Asp	Asp	
		290					295					300					

155

Ser Gly Gln Leu Lys Ser Val Ile Val Gln Arg Ile Tyr Thr Glu Glu
 305 310 315 320

5 Gln Arg Gly Ser Phe Ala Pro Val Thr Ala His Gly Thr Ile Val Val
 325 330 335

Asp Arg Ile Leu Ala Ser Cys Tyr Ala Val Ile Glu Asp Gln Gly Leu
 340 345 350

10 Ala His Leu Ala Phe Ala Pro Ala Arg Leu Tyr Tyr Tyr Val Ser Ser
 355 360 365

Phe Leu Ser Pro Lys Thr Pro Ala Val Gly Pro Met Arg Leu Tyr Asn
 370 375 380

15 Arg Arg Gly Ser Thr Gly Thr Pro Gly Ser Cys His Gln Met Gly Thr
 385 390 395 400

20 Trp Leu Leu Asp Ser Asn Met Leu His Pro Leu Gly Met Ser Val Asn
 405 410 415

Ser Ser

25

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 475 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

Met Leu Leu Leu Ala Arg Cys Leu Leu Leu Val Leu Val Ser Ser Leu
 1 5 10 15

40 Leu Val Cys Ser Gly Leu Ala Cys Gly Pro Gly Arg Gly Phe Gly Lys
 20 25 30

Arg Arg His Pro Lys Lys Leu Thr Pro Leu Ala Tyr Lys Gln Phe Ile
 35 40 45

45 Pro Asn Val Ala Glu Lys Thr Leu Gly Ala Ser Gly Arg Tyr Glu Gly
 50 55 60

50 Lys Ile Ser Arg Asn Ser Glu Arg Phe Lys Glu Leu Thr Pro Asn Tyr
 65 70 75 80

Asn Pro Asp Ile Ile Phe Lys Asp Glu Glu Asn Thr Gly Ala Asp Arg
 85 90 95

55 Leu Met Thr Gln Arg Cys Lys Asp Lys Leu Asn Ala Leu Ala Ile Ser
 100 105 110

156

	Val	Met	Asn	Gln	Trp	Pro	Gly	Val	Lys	Leu	Arg	Val	Thr	Glu	Gly	Trp	115	120	125
5	Asp	Glu	Asp	Gly	His	His	Ser	Glu	Glu	Ser	Leu	His	Tyr	Glu	Gly	Arg	130	135	140
	Ala	Val	Asp	Ile	Thr	Thr	Ser	Asp	Arg	Asp	Arg	Ser	Lys	Tyr	Gly	Met	145	150	155
10	Leu	Ala	Arg	Leu	Ala	Val	Glu	Ala	Gly	Phe	Asp	Trp	Val	Tyr	Tyr	Glu	165	170	175
	Ser	Lys	Ala	His	Ile	His	Cys	Ser	Val	Lys	Ala	Glu	Asn	Ser	Val	Ala	180	185	190
15	Ala	Lys	Ser	Gly	Gly	Cys	Phe	Pro	Gly	Ser	Ala	Thr	Val	His	Leu	Glu	195	200	205
20	Gln	Gly	Gly	Thr	Lys	Leu	Val	Lys	Asp	Leu	Ser	Pro	Gly	Asp	Arg	Val	210	215	220
	Leu	Ala	Ala	Asp	Asp	Gln	Gly	Arg	Leu	Leu	Tyr	Ser	Asp	Phe	Leu	Thr	225	230	235
25	Phe	Leu	Asp	Arg	Asp	Asp	Gly	Ala	Lys	Lys	Val	Phe	Tyr	Val	Ile	Glu	245	250	255
	Thr	Arg	Glu	Pro	Arg	Glu	Arg	Leu	Leu	Leu	Thr	Ala	Ala	His	Leu	Leu	260	265	270
30	Phe	Val	Ala	Pro	His	Asn	Asp	Ser	Ala	Thr	Gly	Glu	Pro	Glu	Ala	Ser	275	280	285
35	Ser	Gly	Ser	Gly	Pro	Pro	Ser	Gly	Gly	Ala	Leu	Gly	Pro	Arg	Ala	Leu	290	295	300
	Phe	Ala	Ser	Arg	Val	Arg	Pro	Gly	Gln	Arg	Val	Tyr	Val	Val	Ala	Glu	305	310	315
40	Arg	Asp	Gly	Asp	Arg	Arg	Leu	Leu	Pro	Ala	Ala	Val	His	Ser	Val	Thr	325	330	335
	Leu	Ser	Glu	Glu	Ala	Ala	Gly	Ala	Tyr	Ala	Pro	Leu	Thr	Ala	Gln	Gly	340	345	350
45	Thr	Ile	Leu	Ile	Asn	Arg	Val	Leu	Ala	Ser	Cys	Tyr	Ala	Val	Ile	Glu	355	360	365
50	Glu	His	Ser	Trp	Ala	His	Arg	Ala	Phe	Ala	Pro	Phe	Arg	Leu	Ala	His	370	375	380
	Ala	Leu	Leu	Ala	Ala	Leu	Ala	Pro	Ala	Arg	Thr	Asp	Arg	Gly	Gly	Asp	385	390	395
55	Ser	Gly	Gly	Gly	Asp	Arg	Gly	Gly	Gly	Gly	Gly	Arg	Val	Ala	Leu	Thr	405	410	415

157

Ala Pro Gly Ala Ala Asp Ala Pro Gly Ala Gly Ala Thr Ala Gly Ile
 420 425 430

5 His Trp Tyr Ser Gln Leu Leu Tyr Gln Ile Gly Thr Trp Leu Leu Asp
 435 440 445

Ser Glu Ala Leu His Pro Leu Gly Met Ala Val Lys Ser Ser Xaa Ser
 450 455 460

10 Arg Gly Ala Gly Gly Gly Ala Arg Glu Gly Ala
 465 470 475

(2) INFORMATION FOR SEQ ID NO:14:

15

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 313 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

20

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

25 Arg Arg Leu Met Thr Gln Arg Cys Lys Asp Arg Leu Asn Ser Leu Ala
 1 5 10 15

Ile Ser Val Met Asn Gln Trp Pro Gly Val Lys Leu Arg Val Thr Glu
 20 25 30

30

Gly Trp Asp Glu Asp Gly His His Ser Glu Glu Ser Leu His Tyr Glu
 35 40 45

35 Gly Arg Ala Val Asp Ile Thr Thr Ser Asp Arg Asp Arg Asn Lys Tyr
 50 55 60

Gly Leu Leu Ala Arg Leu Ala Val Glu Ala Gly Phe Asp Trp Val Tyr
 65 70 75 80

40 Tyr Glu Ser Lys Ala His Val His Cys Ser Val Lys Ser Glu His Ser
 85 90 95

Ala Ala Ala Lys Thr Gly Gly Cys Phe Pro Ala Gly Ala Gln Val Arg
 100 105 110

45

Leu Glu Ser Gly Ala Arg Val Ala Leu Ser Ala Val Arg Pro Gly Asp
 115 120 125

50 Arg Val Leu Ala Met Gly Glu Asp Gly Ser Pro Thr Phe Ser Asp Val
 130 135 140

Leu Ile Phe Leu Asp Arg Glu Pro His Arg Leu Arg Ala Phe Gln Val
 145 150 155 160

55 Ile Glu Thr Gln Asp Pro Pro Arg Arg Leu Ala Leu Thr Pro Ala His
 165 170 175

158

Leu Leu Phe Thr Ala Asp Asn His Thr Glu Pro Ala Ala Arg Phe Arg
 180 185 190

5 Ala Thr Phe Ala Ser His Val Gln Pro Gly Gln Tyr Val Leu Val Ala
 195 200 205

Gly Val Pro Gly Leu Gln Pro Ala Arg Val Ala Ala Val Ser Thr His
 210 215 220

10 Val Ala Leu Gly Ala Tyr Ala Pro Leu Thr Lys His Gly Thr Leu Val
 225 230 235 240

Val Glu Asp Val Val Ala Ser Cys Phe Ala Ala Val Ala Asp His His
 245 250 255

15 Leu Ala Gln Leu Ala Phe Trp Pro Leu Arg Leu Phe His Ser Leu Ala
 260 265 270

20 Trp Gly Ser Trp Thr Pro Gly Glu Gly Val His Trp Tyr Pro Gln Leu
 275 280 285

Leu Tyr Arg Leu Gly Arg Leu Leu Leu Glu Glu Gly Ser Phe His Pro
 290 295 300

25 Leu Gly Met Ser Gly Ala Gly Ser Xaa
 305 310

(2) INFORMATION FOR SEQ ID NO:15:

30 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 64 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

35 (ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: internal

40 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

45 Gln Arg Cys Lys Asp Lys Leu Asn Ser Leu Ala Ile Ser Val Met Asn
 1 5 10 15

His Trp Pro Gly Val Lys Leu Arg Val Thr Glu Gly Trp Asp Glu Asp
 20 25 30

50 Gly His His Phe Glu Glu Ser Leu His Tyr Glu Gly Arg Ala Val Asp
 35 40 45

Ile Thr Thr Ser Asp Arg Asp Lys Ser Lys Tyr Gly Thr Leu Ser Arg
 50 55 60

55

(2) INFORMATION FOR SEQ ID NO:16:

159

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 65 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

Gln Arg Cys Lys Glu Lys Leu Asn Ser Leu Ala Ile Ser Val Met Asn
 1 5 10 15
 Met Trp Pro Gly Val Lys Leu Arg Val Thr Glu Gly Trp Asp Glu Asp
 20 25 30
 Gly Asn His Phe Glu Asp Ser Leu His Tyr Glu Gly Arg Ala Val Asp
 35 40 45
 Ile Thr Thr Ser Ser Asp Arg Asp Arg Asn Lys Tyr Gly Met Phe Ala
 50 55 60
 Arg
 65

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 64 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

Gln Arg Cys Lys Asp Lys Leu Asn Ser Leu Ala Ile Ser Val Met Asn
 1 5 10 15
 Leu Trp Pro Gly Val Lys Leu Arg Val Thr Glu Gly Trp Asp Glu Asp
 20 25 30
 Gly Leu His Ser Glu Glu Ser Leu His Tyr Glu Gly Arg Ala Val Asp
 35 40 45
 Ile Thr Thr Ser Asp Arg Asp Arg Asn Lys Tyr Arg Met Leu Ala Arg
 50 55 60

(2) INFORMATION FOR SEQ ID NO:18: 160

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 38 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

GGAATTCCCA GCAGNTGCTA AAGGAAGCAA GNGCTNAA

38

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 33 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

TCATCGATGG ACCCAGATCG AAANCCNGCT CTC

33

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 29 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

GCTCTAGAGC TCNACNGCNA GANCGTNGC

29

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 50 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

161

5 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

AGCTGTCGAC GCGGCCGCTA CGTAGGTTAC CGACGTCAAG CTTAGATCTC

50

10 (2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 50 base pairs

(B) TYPE: nucleic acid

15 (C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

20

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

25 AGCTGAGATC TAAGCTTGAC GTCGGTAACC TACGTAGCGG CCGCGTCGAC

50

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 45 base pairs

(B) TYPE: nucleic acid

30 (C) STRANDEDNESS: single

(D) TOPOLOGY: linear

35 (ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

40

GATCGGCCAG GCAGGCCTCG CGATATCGTC ACCGCGGTAT TCGAA

45

(2) INFORMATION FOR SEQ ID NO:24:

45

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 30 base pairs

(B) TYPE: nucleic acid

50 (C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

55

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

AGTGCCAGTC GGGGCCCCCA GGGCCGCGCC 162

30

(2) INFORMATION FOR SEQ ID NO:25:

- 5 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 20 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
10 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

15 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

TACCACAGCG GATGGTTCGG

20

20 (2) INFORMATION FOR SEQ ID NO:26:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 20 base pairs
 (B) TYPE: nucleic acid
25 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

30

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

35 GTGGTGGTTA TGCCGATCGC

20

(2) INFORMATION FOR SEQ ID NO:27:

- 40 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 21 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

45 (ii) MOLECULE TYPE: cDNA

50 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

TAAGAGGCCT ATAAGAGGCG G

21

(2) INFORMATION FOR SEQ ID NO:28:

- 55 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 20 base pairs
 (B) TYPE: nucleic acid

(C) STRANDEDNESS: single 163
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

5

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

10 AAGTCAGCCC AGAGGAGACT

20

(2) INFORMATION FOR SEQ ID NO:29:

15 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

20 (ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: internal

25

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

Cys Gly Pro Gly Arg Gly
1 5

30

(2) INFORMATION FOR SEQ ID NO:30:

35 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

40 (ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

45

AGCAGNTGCT AAAGGAAGCA AGNGCTNAA

29

(2) INFORMATION FOR SEQ ID NO:31:

50

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

55

(ii) MOLECULE TYPE: cDNA

164

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

CTCNACNGCN AGANCKNGTN GCNA

24

5

(2) INFORMATION FOR SEQ ID NO:32:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 32 base pairs

10 (B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

15

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

20 CTGCAGGGAT CCACCATGCG GCTTTTGACG AG

32

(2) INFORMATION FOR SEQ ID NO:33:

25 (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 31 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

30

(ii) MOLECULE TYPE: cDNA

35 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

CTGCAGGGAT CCTTATTCCA CACGAGGGAT T

31

40 (2) INFORMATION FOR SEQ ID NO:34:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 471 amino acids

(B) TYPE: amino acid

45 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: internal

50

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:34:

55 Met Asp Asn His Ser Ser Val Pro Trp Ala Ser Ala Ala Ser Val Thr
1 5 10 15

	Cys	Leu	Ser	Leu	Asp	Ala	Lys	Cys	His	Ser	Ser	Ser	Ser	Ser	Ser	Ser	Ser
				20					25						30		
5	Ser	Lys	Ser	Ala	Ala	Ser	Ser	Ile	Ser	Ala	Ile	Pro	Gln	Glu	Glu	Thr	
			35					40					45				
	Gln	Thr	Met	Arg	His	Ile	Ala	His	Thr	Gln	Arg	Cys	Leu	Ser	Arg	Leu	
		50					55					60					
10	Thr	Ser	Leu	Val	Ala	Leu	Leu	Leu	Ile	Val	Leu	Pro	Met	Val	Phe	Ser	
	65					70					75					80	
	Pro	Ala	His	Ser	Cys	Gly	Pro	Gly	Arg	Gly	Leu	Gly	Arg	His	Arg	Ala	
					85					90					95		
15	Arg	Asn	Leu	Tyr	Pro	Leu	Val	Leu	Lys	Gln	Thr	Ile	Pro	Asn	Leu	Ser	
				100					105					110			
	Glu	Tyr	Thr	Asn	Ser	Ala	Ser	Gly	Pro	Leu	Glu	Gly	Val	Ile	Arg	Arg	
20				115				120					125				
	Asp	Ser	Pro	Lys	Phe	Lys	Asp	Leu	Val	Pro	Asn	Tyr	Asn	Arg	Asp	Ile	
		130					135					140					
25	Leu	Phe	Arg	Asp	Glu	Glu	Gly	Thr	Gly	Ala	Asp	Arg	Leu	Met	Ser	Lys	
	145					150					155					160	
	Arg	Cys	Lys	Glu	Lys	Leu	Asn	Val	Leu	Ala	Tyr	Ser	Val	Met	Asn	Glu	
					165					170					175		
30	Trp	Pro	Gly	Ile	Arg	Leu	Leu	Val	Thr	Glu	Ser	Trp	Asp	Glu	Asp	Tyr	
				180					185					190			
	His	His	Gly	Gln	Glu	Ser	Leu	His	Tyr	Glu	Gly	Arg	Ala	Val	Thr	Ile	
35			195					200					205				
	Ala	Thr	Ser	Asp	Arg	Asp	Gln	Ser	Lys	Tyr	Gly	Met	Leu	Ala	Arg	Leu	
		210					215					220					
40	Ala	Val	Glu	Ala	Gly	Phe	Asp	Trp	Val	Ser	Tyr	Val	Ser	Arg	Arg	His	
	225					230					235					240	
	Ile	Tyr	Cys	Ser	Val	Lys	Ser	Asp	Ser	Ser	Ile	Ser	Ser	His	Val	His	
					245					250					255		
45	Gly	Cys	Phe	Thr	Pro	Glu	Ser	Thr	Ala	Leu	Leu	Glu	Ser	Gly	Val	Arg	
				260					265					270			
	Lys	Pro	Leu	Gly	Glu	Leu	Ser	Ile	Gly	Asp	Arg	Val	Leu	Ser	Met	Thr	
50			275					280					285				
	Ala	Asn	Gly	Gln	Ala	Val	Tyr	Ser	Glu	Val	Ile	Leu	Phe	Met	Asp	Arg	
		290					295					300					
55	Asn	Leu	Glu	Gln	Met	Gln	Asn	Phe	Val	Gln	Leu	His	Thr	Asp	Gly	Gly	
	305					310					315					320	

Ala Val Leu Thr Val Thr Pro Ala ¹⁶⁶ His Leu Val Ser Val Trp Gln Pro
 325 330 335
 5 Glu Ser Gln Lys Leu Thr Phe Val Phe Ala Asp Arg Ile Glu Glu Lys
 340 345 350
 Asn Gln Val Leu Val Arg Asp Val Glu Thr Gly Glu Leu Arg Pro Gln
 355 360 365
 10 Arg Val Val Lys Val Gly Ser Val Arg Ser Lys Gly Val Val Ala Pro
 370 375 380
 Leu Thr Arg Glu Gly Thr Ile Val Val Asn Ser Val Ala Ala Ser Cys
 385 390 395 400
 15 Tyr Ala Val Ile Asn Ser Gln Ser Leu Ala His Trp Gly Leu Ala Pro
 405 410 415
 20 Met Arg Leu Leu Ser Thr Leu Glu Ala Trp Leu Pro Ala Lys Glu Gln
 420 425 430
 Leu His Ser Ser Pro Lys Val Val Ser Ser Ala Gln Gln Gln Asn Gly
 435 440 445
 25 Ile His Trp Tyr Ala Asn Ala Leu Tyr Lys Val Lys Asp Tyr Val Leu
 450 455 460
 Pro Gln Ser Trp Arg His Asp
 465 470
 30

(2) INFORMATION FOR SEQ ID NO:35:

35 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 73 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear
 40 (ii) MOLECULE TYPE: peptide
 (v) FRAGMENT TYPE: internal
 45 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:35:
 Arg Cys Lys Glu Arg Val Asn Ser Leu Ala Ile Ala Val Met His Met
 1 5 10 15
 50 Trp Pro Gly Val Arg Leu Arg Val Thr Glu Gly Trp Asp Glu Asp Gly
 20 25 30
 His His Leu Pro Asp Ser Leu His Tyr Glu Gly Arg Ala Leu Asp Ile
 35 40 45
 55 Thr Thr Ser Asp Arg Asp Arg His Lys Tyr Gly Met Leu Ala Arg Leu
 50 55 60

167

Ala Val Glu Ala Gly Phe Asp Trp Val
65 70

5 (2) INFORMATION FOR SEQ ID NO:36:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 73 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:36:

Arg Cys Lys Asp Lys Leu Asn Ala Leu Ala Ile Ser Val Met Asn Gln
1 5 10 15
Trp Pro Gly Val Lys Leu Arg Val Thr Glu Gly Trp Asp Glu Asp Gly
20 25 30
His His Ser Glu Glu Ser Leu His Tyr Glu Gly Arg Ala Val Asp Ile
35 40 45
Thr Thr Ser Asp Arg Asp Arg Ser Lys Tyr Gly Met Leu Ala Arg Leu
50 55 60
Ala Val Glu Ala Gly Phe Asp Trp Val
65 70

(2) INFORMATION FOR SEQ ID NO:37:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 64 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:37:

Lys Arg Cys Lys Glu Lys Leu Asn Val Leu Ala Tyr Ser Val Met Asn
1 5 10 15
Glu Trp Pro Gly Ile Arg Leu Val Val Thr Glu Ser Trp Asp Glu Asp
20 25 30
Tyr His His Gly Gln Glu Ser Leu His Tyr Glu Gly Arg Ala Val Thr
35 40 45

168

Ile	Ala	Thr	Ser	Asp	Arg	Asp	Gln	Ser	Lys	Tyr	Gly	Met	Leu	Ala	Arg
50						55					60				

5 (2) INFORMATION FOR SEQ ID NO:38:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 28 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

10

(ii) MOLECULE TYPE: cDNA

15

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:38:

20 AAAAGCTTTA YTGTYAYGTN GGNATHGG

28

(2) INFORMATION FOR SEQ ID NO:39:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 28 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

25

(ii) MOLECULE TYPE: cDNA

30

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:39:

35

AAGAATTCTA NGCRTTTRTAR TTRTTNGG

28

(2) INFORMATION FOR SEQ ID NO:40:

40

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 165 amino acids
 - (B) TYPE: amino acid
 - (D) TOPOLOGY: linear

45

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: internal

50

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:40:

55

Cys	Gly	Pro	Gly	Arg	Gly	Xaa	Gly	Xaa	Arg	Arg	His	Pro	Lys	Lys	Leu
1				5					10					15	
Thr	Pro	Leu	Ala	Tyr	Lys	Gln	Phe	Ile	Pro	Asn	Val	Ala	Glu	Lys	Thr
		20						25						30	

169

Leu Gly Ala Ser Gly Arg Tyr Glu Gly Lys Ile Xaa Arg Asn Ser Glu
 35 40 45

5 Arg Phe Lys Glu Leu Thr Pro Asn Tyr Asn Pro Asp Ile Ile Phe Lys
 50 55 60

Asp Glu Glu Asn Thr Gly Ala Asp Arg Leu Met Thr Gln Arg Cys Lys
 65 70 75 80

10 Asp Lys Leu Asn Xaa Leu Ala Ile Ser Val Met Asn Xaa Trp Pro Gly
 85 90 95

Val Xaa Leu Arg Val Thr Glu Gly Trp Asp Glu Asp Gly His His Xaa
 100 105 110

15 Glu Glu Ser Leu His Tyr Glu Gly Arg Ala Val Asp Ile Thr Thr Ser
 115 120 125

20 Asp Arg Asp Xaa Ser Lys Tyr Gly Xaa Leu Xaa Arg Leu Ala Val Glu
 130 135 140

Ala Gly Phe Asp Trp Val Tyr Tyr Glu Ser Lys Ala His Ile His Cys
 145 150 155 160

25 Ser Val Lys Ala Glu
 165

(2) INFORMATION FOR SEQ ID NO:41:

30

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 167 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

35

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: internal

40

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:41:

45

Cys Gly Pro Gly Arg Gly Xaa Xaa Xaa Arg Arg Xaa Xaa Xaa Pro Lys
 1 5 10 15

Xaa Leu Xaa Pro Leu Xaa Tyr Lys Gln Phe Xaa Pro Xaa Xaa Xaa Glu
 20 25 30

50

Xaa Thr Leu Gly Ala Ser Gly Xaa Xaa Glu Gly Xaa Xaa Xaa Arg Xaa
 35 40 45

Ser Glu Arg Phe Xaa Xaa Leu Thr Pro Asn Tyr Asn Pro Asp Ile Ile
 50 55 60

55

Phe Lys Asp Glu Glu Asn Xaa Gly Ala Asp Arg Leu Met Thr Xaa Arg
 65 70 75 80

[illegible]

20 (2) INFORMATION FOR SEQ ID NO:42:

25 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 3900 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) **FEATURE:**

(A) NAME/KEY: CDS
(B) LOCATION: 1..3897

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:42:

ATG GAC CGC GAC AGC CTC CCA CGC GTT CCG GAC ACA CAC GGC GAT GTG
Met Asp Arg Asp Ser Leu Pro Arg Val Pro Asp Thr His Gly Asp Val

40 1 5 10 15

GTC	GAT	GAG	AAA	TTA	TTC	TCG	GAT	CTT	TAC	ATA	CGC	ACC	AGC	TGG	GTG	96
Val	Asp	Glu	Lys	Leu	Phe	Ser	Asp	Leu	Tyr	Ile	Arg	Thr	Ser	Trp	Val	
			20					25					30			

GAC GCC CAA GTG GCG CTC GAT CAG ATA GAT AAG GGC AAA GCG CGT GGC 144
Asp Ala Gln Val Ala Leu Asp Gln Ile Asp Lys Gly Lys Ala Arg Gly
35 40 45

50 AGC CGC ACG GCG ATC TAT CTG CGA TCA GTA TTC CAG TCC CAC CTC GAA 192
Ser Arg Thr Ala Ile Tyr Leu Arg Ser Val Phe Gln Ser His Leu Glu
 50 55 60

55 ACC CTC GGC AGC TCC GTG CAA AAG CAC GCG GGC AAG GTG CTA TTC GTG 240
Thr Leu Gly Ser Ser Val Gln Lys His Ala Gly Lys Val Leu Phe Val
65 70 75 80

		GCT	ATC	CTG	GTG	CTG	AGC	ACC	TTC	TGC	171 GTC	GGC	CTG	AAG	AGC	GCC	CAG	288
		Ala	Ile	Leu	Val	Leu	Ser	Thr	Phe	Cys	Val	Gly	Leu	Lys	Ser	Ala	Gln	
					85						90					95		
5		ATC	CAC	TCC	AAG	GTG	CAC	CAG	CTG	TGG	ATC	CAG	GAG	GGC	GGC	GGG	CTG	336
		Ile	His	Ser	Lys	Val	His	Gln	Leu	Trp	Ile	Gln	Glu	Gly	Gly	Gly	Leu	
					100					105					110			
10		GAG	GCG	GAA	CTG	GCC	TAC	ACA	CAG	AAG	ACG	ATC	GGC	GAG	GAC	GAG	TCG	384
		Glu	Ala	Glu	Leu	Ala	Tyr	Thr	Gln	Lys	Thr	Ile	Gly	Glu	Asp	Glu	Ser	
					115				120					125				
15		GCC	ACG	CAT	CAG	CTG	CTC	ATT	CAG	ACG	ACC	CAC	GAC	CCG	AAC	GCC	TCC	432
		Ala	Thr	His	Gln	Leu	Leu	Ile	Gln	Thr	Thr	His	Asp	Pro	Asn	Ala	Ser	
					130				135					140				
20		GTC	CTG	CAT	CCG	CAG	GCG	CTG	CTT	GCC	CAC	CTG	GAG	GTC	CTG	GTC	AAG	480
		Val	Leu	His	Pro	Gln	Ala	Leu	Leu	Ala	His	Leu	Glu	Val	Leu	Val	Lys	
							150					155					160	
		GCC	ACC	GCC	GTC	AAG	GTG	CAC	CTC	TAC	GAC	ACC	GAA	TGG	GGG	CTG	CGC	528
		Ala	Thr	Ala	Val	Lys	Val	His	Leu	Tyr	Asp	Thr	Glu	Trp	Gly	Leu	Arg	
						165					170					175		
25		GAC	ATG	TGC	AAC	ATG	CCG	AGC	ACG	CCC	TCC	TTC	GAG	GGC	ATC	TAC	TAC	576
		Asp	Met	Cys	Asn	Met	Pro	Ser	Thr	Pro	Ser	Phe	Glu	Gly	Ile	Tyr	Tyr	
					180					185					190			
30		ATC	GAG	CAG	ATC	CTG	CGC	CAC	CTC	ATT	CCG	TGC	TCG	ATC	ATC	ACG	CCG	624
		Ile	Glu	Gln	Ile	Leu	Arg	His	Leu	Ile	Pro	Cys	Ser	Ile	Ile	Thr	Pro	
					195				200					205				
35		CTG	GAC	TGT	TTC	TGG	GAG	GGA	AGC	CAG	CTG	TTG	GGT	CCG	GAA	TCA	GCG	672
		Leu	Asp	Cys	Phe	Trp	Glu	Gly	Ser	Gln	Leu	Leu	Gly	Pro	Glu	Ser	Ala	
					210				215					220				
40		GTC	GTT	ATA	CCA	GGC	CTC	AAC	CAA	CGA	CTC	CTG	TGG	ACC	ACA	CTG	AAT	720
		Val	Val	Ile	Pro	Gly	Leu	Asn	Gln	Arg	Leu	Leu	Trp	Thr	Thr	Leu	Asn	
							230					235					240	
		CCC	GCC	TCT	GTG	ATG	CAG	TAT	ATG	AAG	CAG	AAG	ATG	TCC	GAG	GAA	AAG	768
		Pro	Ala	Ser	Val	Met	Gln	Tyr	Met	Lys	Gln	Lys	Met	Ser	Glu	Glu	Lys	
						245					250					255		
45		ATC	AGC	TTC	GAC	TTC	GAG	ACC	GTG	GAG	CAG	TAC	ATG	AAG	CGT	GCG	GCC	816
		Ile	Ser	Phe	Asp	Phe	Glu	Thr	Val	Glu	Gln	Tyr	Met	Lys	Arg	Ala	Ala	
					260					265					270			
50		ATT	GCG	AGT	GGC	TAC	ATG	GAG	AAG	CCC	TGC	CTG	AAC	CCA	CTG	AAT	CCC	864
		Ile	Ala	Ser	Gly	Tyr	Met	Glu	Lys	Pro	Cys	Leu	Asn	Pro	Leu	Asn	Pro	
					275				280					285				
55		AAT	TGC	CCG	GAC	ACG	GCA	CCG	AAC	AAG	AAC	AGC	ACC	CAG	CCG	CCG	GAT	912
		Asn	Cys	Pro	Asp	Thr	Ala	Pro	Asn	Lys	Asn	Ser	Thr	Gln	Pro	Pro	Asp	
					290				295					300				

	GTG	GGA	GCC	ATC	CTG	TCC	GGA	GGC	TGC	TAC	GGT	TAT	GCC	GCG	AAG	CAC	
	Val	Gly	Ala	Ile	Leu	Ser	Gly	Gly	Cys	Tyr	Gly	Tyr	Ala	Ala	Lys	His	960
	305					310					315					320	
5	ATG	CAC	TGG	CCG	GAG	GAG	CTG	ATT	GTG	GGC	GGA	GCG	AAG	AGG	AAC	CGC	1008
	Met	His	Trp	Pro	Glu	Glu	Leu	Ile	Val	Gly	Gly	Ala	Lys	Arg	Asn	Arg	
					325					330					335		
10	AGC	GGA	CAC	TTG	AGG	AAG	GCC	CAG	GCC	CTG	CAG	TCG	GTG	GTG	CAG	CTG	1056
	Ser	Gly	His	Leu	Arg	Lys	Ala	Gln	Ala	Leu	Gln	Ser	Val	Val	Gln	Leu	
				340					345					350			
15	ATG	ACC	GAG	AAG	GAA	ATG	TAC	GAC	CAG	TGG	CAG	GAC	AAC	TAC	AAG	GTG	1104
	Met	Thr	Glu	Lys	Glu	Met	Tyr	Asp	Gln	Trp	Gln	Asp	Asn	Tyr	Lys	Val	
			355					360					365				
20	CAC	CAT	CTT	GGA	TGG	ACG	CAG	GAG	AAG	GCA	GCG	GAG	GTT	TTG	AAC	GCC	1152
	His	His	Leu	Gly	Trp	Thr	Gln	Glu	Lys	Ala	Ala	Glu	Val	Leu	Asn	Ala	
		370					375					380					
25	TGG	CAG	CGC	AAC	TTT	TCG	CGG	GAG	GTG	GAA	CAG	CTG	CTA	CGT	AAA	CAG	1200
	Trp	Gln	Arg	Asn	Phe	Ser	Arg	Glu	Val	Glu	Gln	Leu	Leu	Arg	Lys	Gln	
	385					390					395					400	
30	TCG	AGA	ATT	GCC	ACC	AAC	TAC	GAT	ATC	TAC	GTG	TTC	AGC	TCG	GCT	GCA	1248
	Ser	Arg	Ile	Ala	Thr	Asn	Tyr	Asp	Ile	Tyr	Val	Phe	Ser	Ser	Ala	Ala	
				405					410						415		
35	CTG	GAT	GAC	ATC	CTG	GCC	AAG	TTC	TCC	CAT	CCC	AGC	GCC	TTG	TCC	ATT	1296
	Leu	Asp	Asp	Ile	Leu	Ala	Lys	Phe	Ser	His	Pro	Ser	Ala	Leu	Ser	Ile	
				420					425					430			
40	GTC	ATC	GGC	GTG	GCC	GTC	ACC	GTT	TTG	TAT	GCC	TTC	TGC	ACG	CTC	CTC	1344
	Val	Ile	Gly	Val	Ala	Val	Thr	Val	Leu	Tyr	Ala	Phe	Cys	Thr	Leu	Leu	
			435					440					445				
45	CGC	TGG	AGG	GAC	CCC	GTC	CGT	GGA	CAG	AGC	AGT	GTC	GGC	GTG	GCC	GGA	1392
	Arg	Trp	Arg	Asp	Pro	Val	Arg	Gly	Gln	Ser	Ser	Val	Gly	Val	Ala	Gly	
		450					455					460					
50	GTT	CTG	CTC	ATG	TGC	TTT	AGT	ACC	GCC	GCC	GGA	TTG	GGA	TTG	TCA	GCC	1440
	Val	Leu	Leu	Met	Cys	Phe	Ser	Thr	Ala	Ala	Gly	Leu	Gly	Leu	Ser	Ala	
	465					470					475					480	
55	CTG	CTC	GGT	ATC	GTT	TTC	AAT	GCC	GCC	AGC	ACC	CAG	GTG	GTT	CCG	TTT	1488
	Leu	Leu	Gly	Ile	Val	Phe	Asn	Ala	Ala	Ser	Thr	Gln	Val	Val	Pro	Phe	
				485					490						495		
60	TTG	GCC	CTT	GGT	CTG	GGC	GTC	GAT	CAC	ATC	TTC	ATG	CTG	ACC	GCT	GCC	1536

	GTG	GGA	CCG	AGC	ATC	CTG	TTC	AGT	GCC	TGC	AGC	ACC	GCA	GGA	TCC	TTC	1632
	Val	Gly	Pro	Ser	Ile	Leu	Phe	Ser	Ala	Cys	Ser	Thr	Ala	Gly	Ser	Phe	
	530						535					540					
5	TTT	GCG	GCC	GCC	TTT	ATT	CCG	GTG	CCG	GCT	TTG	AAG	GTA	TTC	TGT	CTG	1680
	Phe	Ala	Ala	Ala	Phe	Ile	Pro	Val	Pro	Ala	Leu	Lys	Val	Phe	Cys	Leu	
	545					550					555					560	
10	CAG	GCT	GCC	ATC	GTA	ATG	TGC	TCC	AAT	TTG	GCA	GCG	GCT	CTA	TTG	GTT	1728
	Gln	Ala	Ala	Ile	Val	Met	Cys	Ser	Asn	Leu	Ala	Ala	Ala	Leu	Leu	Val	
					565					570					575		
15	TTT	CCG	GCC	ATG	ATT	TCG	TTG	GAT	CTA	CGG	AGA	CGT	ACC	GCC	GGC	AGG	1776
	Phe	Pro	Ala	Met	Ile	Ser	Leu	Asp	Leu	Arg	Arg	Arg	Thr	Ala	Gly	Arg	
				580					585					590			
20	GCG	GAC	ATC	TTC	TGC	TGC	TGT	TTT	CCG	GTG	TGG	AAG	GAA	CAG	CCG	AAG	1824
	Ala	Asp	Ile	Phe	Cys	Cys	Cys	Phe	Pro	Val	Trp	Lys	Glu	Gln	Pro	Lys	
				595				600					605				
	GTG	GCA	CCA	CCG	GTG	CTG	CCG	CTG	AAC	AAC	AAC	AAC	GGG	CGC	GGG	GCC	1872
	Val	Ala	Pro	Pro	Val	Leu	Pro	Leu	Asn	Asn	Asn	Asn	Gly	Arg	Gly	Ala	
	610						615					620					
25	CGG	CAT	CCG	AAG	AGC	TGC	AAC	AAC	AAC	AGG	GTG	GCG	CTG	CCC	GCC	CAG	1920
	Arg	His	Pro	Lys	Ser	Cys	Asn	Asn	Asn	Arg	Val	Ala	Leu	Pro	Ala	Gln	
	625					630					635					640	
30	AAT	CCT	CTG	CTG	GAA	CAG	AGG	GCA	GAC	ATC	CCT	GGG	AGC	AGT	CAC	TCA	1968
	Asn	Pro	Leu	Leu	Glu	Gln	Arg	Ala	Asp	Ile	Pro	Gly	Ser	Ser	His	Ser	
					645					650					655		
35	CTG	GCG	TCC	TTC	TCT	CTG	GCA	ACA	TTC	GCC	TTT	CAG	CAC	TAC	ACT	CCC	2016
	Leu	Ala	Ser	Phe	Ser	Leu	Ala	Thr	Phe	Ala	Phe	Gln	His	Tyr	Thr	Pro	
				660					665					670			
40	TTC	CTC	ATG	CGC	AGC	TGG	GTG	AAG	TTC	CTG	ACC	GTT	ATG	GGT	TTC	CTG	2064
	Phe	Leu	Met	Arg	Ser	Trp	Val	Lys	Phe	Leu	Thr	Val	Met	Gly	Phe	Leu	
			675					680					685				
	GCG	GCC	CTC	ATA	TCC	AGC	TTG	TAT	GCC	TCC	ACG	CGC	CTT	CAG	GAT	GGC	2112
	Ala	Ala	Leu	Ile	Ser	Ser	Leu	Tyr	Ala	Ser	Thr	Arg	Leu	Gln	Asp	Gly	
	690						695					700					
45	CTG	GAC	ATT	ATT	GAT	CTG	GTG	CCC	AAG	GAC	AGC	AAC	GAG	CAC	AAG	TTC	2160
	Leu	Asp	Ile	Ile	Asp	Leu	Val	Pro	Lys	Asp	Ser	Asn	Glu	His	Lys	Phe	
	705					710					715					720	
50	CTG	GAT	GCT	CAA	ACT	CGG	CTC	TTT	GGC	TTC	TAC	AGC	ATG	TAT	GCG	GTT	2208
	Leu	Asp	Ala	Gln	Thr	Arg	Leu	Phe	Gly	Phe	Tyr	Ser	Met	Tyr	Ala	Val	
					725					730					735		
55	ACC	CAG	GGC	AAC	TTT	GAA	TAT	CCC	ACC	CAG	CAG	CAG	TTG	CTC	AGG	GAC	2256
	Thr	Gln	Gly	Asn	Phe	Glu	Tyr	Pro	Thr	Gln	Gln	Gln	Leu	Leu	Arg	Asp	
				740					745					750			

									174										
	TAC	CAT	GAT	TCC	TTT	GTG	CGG	GTG	CCA	CAT	GTG	ATC	AAG	AAT	GAT	AAT		2304	
	Tyr	His	Asp	Ser	Phe	Val	Arg	Val	Pro	His	Val	Ile	Lys	Asn	Asp	Asn			
			755					760					765						
5	GGT	GGA	CTG	CCG	GAC	TTC	TGG	CTG	CTG	CTC	TTC	AGC	GAG	TGG	CTG	GGT		2352	
	Gly	Gly	Leu	Pro	Asp	Phe	Trp	Leu	Leu	Leu	Phe	Ser	Glu	Trp	Leu	Gly			
		770					775					780							
10	AAT	CTG	CAA	AAG	ATA	TTC	GAC	GAG	GAA	TAC	CGC	GAC	GGA	CGG	CTG	ACC		2400	
	Asn	Leu	Gln	Lys	Ile	Phe	Asp	Glu	Glu	Tyr	Arg	Asp	Gly	Arg	Leu	Thr			
	785					790					795					800			
15	AAG	GAG	TGC	TGG	TTC	CCA	AAC	GCC	AGC	AGC	GAT	GCC	ATC	CTG	GCC	TAC		2448	
	Lys	Glu	Cys	Trp	Phe	Pro	Asn	Ala	Ser	Ser	Asp	Ala	Ile	Leu	Ala	Tyr			
					805					810					815				
20	AAG	CTA	ATC	GTG	CAA	ACC	GGC	CAT	GTG	GAC	AAC	CCC	GTG	GAC	AAG	GAA		2496	
	Lys	Leu	Ile	Val	Gln	Thr	Gly	His	Val	Asp	Asn	Pro	Val	Asp	Lys	Glu			
				820					825						830				
	CTG	GTG	CTC	ACC	AAT	CGC	CTG	GTC	AAC	AGC	GAT	GGC	ATC	ATC	AAC	CAA		2544	
	Leu	Val	Leu	Thr	Asn	Arg	Leu	Val	Asn	Ser	Asp	Gly	Ile	Ile	Asn	Gln			
			835					840						845					
25	CGC	GCC	TTC	TAC	AAC	TAT	CTG	TCG	GCA	TGG	GCC	ACC	AAC	GCG	TCT	TCG		2592	
	Arg	Ala	Phe	Tyr	Asn	Tyr	Leu	Ser	Ala	Trp	Ala	Thr	Asn	Ala	Ser	Ser			
		850					855					860							
30	CCT	ACG	GAG	CTT	CTC	AGG	GCA	AAT	TGT	ATC	CGG	AAC	CGC	GCC	AAC	GGA		2640	
	Pro	Thr	Glu	Leu	Leu	Arg	Ala	Asn	Cys	Ile	Arg	Asn	Arg	Ala	Asn	Gly			
	865					870					875					880			
35	GCT	TCT	CAG	GGC	AAA	TTG	TAT	CCG	GAA	CCG	CGC	CAG	TAT	TTT	CAC	CAA		2688	
	Ala	Ser	Gln	Gly	Lys	Leu	Tyr	Pro	Glu	Pro	Arg	Gln	Tyr	Phe	His	Gln			
					885					890					895				
40	CCC	AAC	GAG	TAC	GAT	CTT	AAG	ATA	CCC	AAG	AGT	CTG	CCA	TTG	GTC	TAC		2736	
	Pro	Asn	Glu	Tyr	Asp	Leu	Lys	Ile	Pro	Lys	Ser	Leu	Pro	Leu	Val	Tyr			
				900					905						910				
	GCT	CAG	ATG	CCC	TTT	TAC	CTC	CAC	GGA	CTA	ACA	GAT	ACC	TCG	CAG	ATC		2784	
	Ala	Gln	Met	Pro	Phe	Tyr	Leu	His	Gly	Leu	Thr	Asp	Thr	Ser	Gln	Ile			
			915					920					925						
45	AAG	ACC	CTG	ATA	GGT	CAT	ATT	CGC	GAC	CTG	AGC	GTC	AAG	TAC	GAG	GGC		2832	
	Lys	Thr	Leu	Ile	Gly	His	Ile	Arg	Asp	Leu	Ser	Val	Lys	Tyr	Glu	Gly			
		930					935					940							
50	TTC	GGC	CTG	CCC	AAC	TAT	CCA	TCG	GGC	ATT	CCC	TTC	ATC	TTC	TGG	GAG		2880	
	Phe	Gly	Leu	Pro	Asn	Tyr	Pro	Ser	Gly	Ile	Pro	Phe	Ile	Phe	Trp	Glu			
	945					950					955					960			
55	CAG	TAC	ATG	ACC	CTG	CGC	TCC	TCA	CTG	GCC	ATG	ATC	CTG	GCC	TGC	GTG		2928	
	Gln	Tyr	Met	Thr	Leu	Arg	Ser	Ser	Leu	Ala	Met	Ile	Leu	Ala	Cys	Val			
					965					970						975			

175

	CTA	CTC	GCC	GCC	CTG	GTG	CTG	GTC	TCC	CTG	CTC	CTG	CTC	TCC	GTT	TGG	2976
	Leu	Leu	Ala	Ala	Leu	Val	Leu	Val	Ser	Leu	Leu	Leu	Leu	Ser	Val	Trp	
				980					985					990			
5	GCC	GCC	GTT	CTC	GTG	ATC	CTC	AGC	GTT	CTG	GCC	TCG	CTG	GCC	CAG	ATC	3024
	Ala	Ala	Val	Leu	Val	Ile	Leu	Ser	Val	Leu	Ala	Ser	Leu	Ala	Gln	Ile	
			995					1000					1005				
10	TTT	GGG	GCC	ATG	ACT	CTG	CTG	GGC	ATC	AAA	CTC	TCG	GCC	ATT	CCG	GCA	3072
	Phe	Gly	Ala	Met	Thr	Leu	Leu	Gly	Ile	Lys	Leu	Ser	Ala	Ile	Pro	Ala	
		1010						1015					1020				
15	GTC	ATA	CTC	ATC	CTC	AGC	GTG	GGC	ATG	ATG	CTG	TGC	TTC	AAT	GTG	CTG	3120
	Val	Ile	Leu	Ile	Leu	Ser	Val	Gly	Met	Met	Leu	Cys	Phe	Asn	Val	Leu	
		1025					1030				1035					1040	
20	ATA	TCA	CTG	GGC	TTC	ATG	ACA	TCC	GTT	GGC	AAC	CGA	CAG	CGC	CGC	GTC	3168
	Ile	Ser	Leu	Gly	Phe	Met	Thr	Ser	Val	Gly	Asn	Arg	Gln	Arg	Arg	Val	
					1045					1050					1055		
	CAG	CTG	AGC	ATG	CAG	ATG	TCC	CTG	GGA	CCA	CTT	GTC	CAC	GGC	ATG	CTG	3216
	Gln	Leu	Ser	Met	Gln	Met	Ser	Leu	Gly	Pro	Leu	Val	His	Gly	Met	Leu	
				1060					1065					1070			
25	ACC	TCC	GGA	GTG	GCC	GTG	TTC	ATG	CTC	TCC	ACG	TCG	CCC	TTT	GAG	TTT	3264
	Thr	Ser	Gly	Val	Ala	Val	Phe	Met	Leu	Ser	Thr	Ser	Pro	Phe	Glu	Phe	
			1075					1080						1085			
30	GTG	ATC	CGG	CAC	TTC	TGC	TGG	CTT	CTG	CTG	GTG	GTC	TTA	TGC	GTT	GGC	3312
	Val	Ile	Arg	His	Phe	Cys	Trp	Leu	Leu	Leu	Val	Val	Leu	Cys	Val	Gly	
		1090					1095						1100				
35	GCC	TGC	AAC	AGC	CTT	TTG	GTG	TTC	CCC	ATC	CTA	CTG	AGC	ATG	GTG	GGA	3360
	Ala	Cys	Asn	Ser	Leu	Leu	Val	Phe	Pro	Ile	Leu	Leu	Ser	Met	Val	Gly	
		1105					1110				1115					1120	
40	CCG	GAG	GCG	GAG	CTG	GTG	CCG	CTG	GAG	CAT	CCA	GAC	CGC	ATA	TCC	ACG	3408
	Pro	Glu	Ala	Glu	Leu	Val	Pro	Leu	Glu	His	Pro	Asp	Arg	Ile	Ser	Thr	
					1125					1130					1135		
	CCC	TCT	CCG	CTG	CCC	GTG	CGC	AGC	AGC	AAG	AGA	TCG	GGC	AAA	TCC	TAT	3456
	Pro	Ser	Pro	Leu	Pro	Val	Arg	Ser	Ser	Lys	Arg	Ser	Gly	Lys	Ser	Tyr	
				1140					1145					1150			
45	GTG	GTG	CAG	GGA	TCG	CGA	TCC	TCG	CGA	GGC	AGC	TGC	CAG	AAG	TCG	CAT	3504
	Val	Val	Gln	Gly	Ser	Arg	Ser	Ser	Arg	Gly	Ser	Cys	Gln	Lys	Ser	His	
			1155					1160					1165				
50	CAC	CAC	CAC	CAC	AAA	GAC	CTT	AAT	GAT	CCA	TCG	CTG	ACG	ACG	ATC	ACC	3552
	His	His	His	His	Lys	Asp	Leu	Asn	Asp	Pro	Ser	Leu	Thr	Thr	Ile	Thr	
			1170				1175					1180					
55	GAG	GAG	CCG	CAG	TCG	TGG	AAG	TCC	AGC	AAC	TCG	TCC	ATC	CAG	ATG	CCC	3600
	Glu	Glu	Pro	Gln	Ser	Trp	Lys	Ser	Ser	Asn	Ser	Ser	Ile	Gln	Met	Pro	
		1185					1190				1195					1200	

176

	AAT GAT TGG ACC TAC CAG CCG CGG GAA CAG CGA CCC GCC TCC TAC GCG	3648
	Asn Asp Trp Thr Tyr Gln Pro Arg Glu Gln Arg Pro Ala Ser Tyr Ala	
	1205 1210 1215	
5	GCC CCG CCC CCC GCC TAT CAC AAG GCC GCC GCC CAG CAG CAC CAC CAG	3696
	Ala Pro Pro Pro Ala Tyr His Lys Ala Ala Ala Gln Gln His His Gln	
	1220 1225 1230	
10	CAT CAG GGC CCG CCC ACA ACG CCC CCG CCG CCC TTC CCG ACG GCC TAT	3744
	His Gln Gly Pro Pro Thr Thr Pro Pro Pro Phe Pro Thr Ala Tyr	
	1235 1240 1245	
15	CCG CCG GAG CTG CAG AGC ATC GTG GTG CAG CCG GAG GTG ACG GTG GAG	3792
	Pro Pro Glu Leu Gln Ser Ile Val Val Gln Pro Glu Val Thr Val Glu	
	1250 1255 1260	
20	ACG ACG CAC TCG GAC AGC AAC ACC ACC AAG GTG ACG GCC ACG GCC AAC	3840
	Thr Thr His Ser Asp Ser Asn Thr Thr Lys Val Thr Ala Thr Ala Asn	
	1265 1270 1275 1280	
	ATC AAG GTG GAG CTG GCC ATG CCC GGC AGG GCG GTG CGC AGC TAT AAC	3888
	Ile Lys Val Glu Leu Ala Met Pro Gly Arg Ala Val Arg Ser Tyr Asn	
	1285 1290 1295	
25	TTT ACG AGT TAG	3900
	Phe Thr Ser	

(2) INFORMATION FOR SEQ ID NO:43:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 24 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:43:

ACCGAGGGCT GGGACGAAGA TGGC

24

(2) INFORMATION FOR SEQ ID NO:44:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 25 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

177

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:44:

CGCTCGGTCG TACGGCATGA ACGAC

25

5

(2) INFORMATION FOR SEQ ID NO:45:

(i) SEQUENCE CHARACTERISTICS:

10

(A) LENGTH: 27 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

15

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:45:

20

ATGGGGATGT GTGTGTGGTC AAGTGTA

27

(2) INFORMATION FOR SEQ ID NO:46:

25

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 25 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

30

(ii) MOLECULE TYPE: cDNA

35

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:46:

TTCACAGACT CTCAAAGTGT ATTTT

25

40

(2) INFORMATION FOR SEQ ID NO:47:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

45

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: internal

50

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:47:

55

Met Gly Ser Ser His His His His His Leu Val Pro Arg Gly Ser
1 5 10 15

His Met

178

What is claimed is:

1. A substantially pure preparation of a *hedgehog*-like protein comprising a polypeptide having an amino acid sequence identical or homologous to a vertebrate *hedgehog* protein and not identical to SEQ ID No. 34.
5
2. The *hedgehog*-like protein of claim 1, which protein is a *Sonic hedgehog* protein, comprising a polypeptide having an amino acid sequence identical or homologous with a polypeptide represented in SEQ ID No. 1, SEQ ID No. 4, SEQ ID No. 5 or SEQ ID No. 6.
10
3. The *hedgehog*-like protein of claim 2, which protein comprises a polypeptide represented by SEQ ID No. 40.
- 15 4. The *hedgehog*-like protein of claim 1, which protein is an *Indian hedgehog* protein, comprising a polypeptide having an amino acid sequence identical or homologous with a polypeptide represented in SEQ ID No. 3 or SEQ ID No. 7.
- 20 5. The *hedgehog*-like protein of claim 1, which protein is a *Desert hedgehog* protein, comprising a polypeptide having an amino acid sequence identical or homologous with the polypeptide represented in SEQ ID No. 2.
- 25 6. The *hedgehog*-like protein of claim 1, which protein comprises a polypeptide represented by SEQ ID No. 41.
7. The *hedgehog*-like protein of claim 1, which protein modulates at least one of proliferation, differentiation or survival of mesodermal or ectodermal cells.
- 30 8. The *hedgehog*-like protein of claim 7, which protein has a biological activity selected from the group consisting of a neuronal differentiation inducing activity, a neuronal survival promoting activity, a limb patterning activity, a stem cell/progenitor cell inducing activity, a germ cell inducing activity, and a combination thereof.
- 35 9. The *hedgehog*-like protein of claim 7, which protein agonizes at least one of proliferation, differentiation or survival of mesodermal or ectodermal cells.
10. The *hedgehog*-like protein of claim 7, which protein antagonizes at least one of proliferation, differentiation or survival of mesodermal or ectodermal cells.

179

11. The *hedgehog*-like protein of claim 1, which protein is glycosylated.
12. The *hedgehog*-like protein of claim 1, which protein comprises an N-terminal portion of
5 a vertebrate *hedgehog* protein having an approximate molecular weight of 19kd.
13. The *hedgehog*-like protein of claim 1, which protein comprises an C-terminal portion of
a vertebrate *hedgehog* protein having an approximate molecular weight of 27kd.
- 10 14. An immunogen comprising a portion of the protein of claim 1, said immunogen being
antigenically distinct from any portion of the protein of SEQ ID No:34.
- 15 15. An antibody preparation specifically reactive with an epitope of the *hedgehog*-like
protein of claim 1.
16. A recombinant protein comprising a *hedgehog* polypeptide sequence identical or
homologous to a vertebrate *hedgehog* protein, or a portion thereof, which recombinant
protein functions in one of either role of an agonist of at least one biological activity of
said vertebrate *hedgehog* protein or an antagonist of at least one biological activity of
20 said vertebrate *hedgehog* protein, said *hedgehog* polypeptide sequence distinct from
SEQ ID No:34.
17. The recombinant protein of claim 16, which *hedgehog* amino acid sequence is at least
25 60 percent homologous with an amino acid sequence of a vertebrate *hedgehog* protein
represented in the group consisting SEQ ID No:8, SEQ ID No:9, SEQ ID No:10, SEQ
ID No:11, SEQ ID No:12, SEQ ID No:13 and SEQ ID No:14.
18. The recombinant protein of claim 16, which *hedgehog* amino acid sequence comprises
an N-terminal portion of a vertebrate *hedgehog* protein corresponding approximately to
30 Cys-24 through Glu-188 of SEQ ID No. 13.
19. The recombinant protein of claim 16, which *hedgehog* amino acid sequence comprises
a C-terminal portion of a vertebrate *hedgehog* protein corresponding approximately to
Asn-189 through Ala-475 of SEQ ID No. 13.
35
20. The recombinant protein of claim 16, which protein is a fusion protein further
comprising, in addition to said *hedgehog* polypeptide sequence, a second polypeptide

180

sequence having an amino acid sequence different than said vertebrate *hedgehog* protein.

- 5 21. The recombinant protein of claim 20, wherein said second polypeptide is a detectable label for detecting the presence of said fusion protein or as a matrix-binding domain for immobilizing said fusion protein.
- 10 22. A recombinant *hedgehog* polypeptide comprising an amino acid sequence at least 70 percent homology with a *hedgehog* amino acid sequence selected from a group consisting of residues 27-425 of SEQ ID No:8, residues 22-396 of SEQ ID No:9, residues 1-336 of SEQ ID No:10, residues 25-437 of SEQ ID No:11, residues 24-418 of SEQ ID No:12, residues 24-475 of SEQ ID No:13, and residues 1-312 of SEQ ID No:14.
- 15 23. An isolated or recombinant *hedgehog* polypeptide having an amino acid sequence crossreactive with an antibody which specifically binds a *hedgehog* protein having an amino acid sequence selected from the group consisting of SEQ ID No:8, SEQ ID No:9, SEQ ID No:10, SEQ ID No:11, SEQ ID No:12, SEQ ID No:13 and SEQ ID No:14.
- 20 24. An isolated or recombinant *hedgehog* polypeptide encoded by a nucleic acid which hybridizes under stringent conditions to a nucleic acid sequence selected from the group consisting of SEQ ID No:1, SEQ ID No:2, SEQ ID No:3, SEQ ID No:4, SEQ ID No:5, SEQ ID No:6 and SEQ ID No:7.
- 25 25. An isolated nucleic acid encoding a *hedgehog* polypeptide having an amino acid sequence identical or homologous to a vertebrate *hedgehog* protein, or a portion thereof, and not identical to SEQ ID No. 34.
- 30 26. The nucleic acid of claim 25, which nucleic acid encodes a *hedgehog* polypeptide that modulates at least one of proliferation, differentiation or survival of mesodermal or ectodermal cells.
- 35 27. The nucleic acid of claim 25, which nucleic acid hybridizes under stringent conditions to a nucleic acid probe having a sequence represented by at least 12 consecutive nucleotides of a *hedgehog* nucleic acid sequence selected from the group consisting of SEQ ID No:1, SEQ ID No:2, SEQ ID No:3, SEQ ID No:4, SEQ ID No:5, SEQ ID No:6 and SEQ ID No:7.

18/

28. The nucleic acid of claim 25, further comprising a transcriptional regulatory sequence operably linked to said nucleotide sequence so as to render said nucleic acid suitable for use as an expression vector.
29. An expression vector, capable of replicating in at least one of a prokaryotic cell and eukaryotic cell, comprising the nucleic acid of claim 25.
30. A host cell transfected with the expression vector of claim 29 and expressing said *hedgehog* polypeptide.
31. A method of producing a recombinant *hedgehog* polypeptide comprising culturing the cell of claim 30 in a cell culture medium to express said *hedgehog* polypeptide and isolating said *hedgehog* polypeptide from said cell culture.
32. A transgenic animal having cells which harbor a transgene comprising the nucleic acid of claim 25.
33. A recombinant transfection system, comprising
- (i) a gene construct including the nucleic acid of claim 25 and operably linked to a transcriptional regulatory sequence for causing expression of said *hedgehog* polypeptide in eukaryotic cells, and
 - (ii) a gene delivery composition for delivering said gene construct to a cell and causing the cell to be transfected with said gene construct.
34. The recombinant transfection system of claim 33, wherein the gene delivery composition is selected from a group consisting of a recombinant viral particle, a liposome, and a poly-cationic nucleic acid binding agent,
35. A transgenic vertebrate animal in which *hedgehog* inductive pathways are inhibited in one or more tissue of said animal by one of either expression of an antagonistic *hedgehog* polypeptide or disruption of a *hedgehog* gene.
36. A probe/primer comprising a substantially purified oligonucleotide, said oligonucleotide containing a region of nucleotide sequence which hybridizes under stringent conditions to at least 15 consecutive nucleotides of sense or antisense sequence of SEQ ID No:1, SEQ ID No:2, SEQ ID No:3, SEQ ID No:4, SEQ ID No:5, SEQ ID No:6, SEQ ID No:7, or naturally occurring mutants thereof.

182

37. The probe/primer of claim 36, which probe/primer further comprises a label group attached thereto and able to be detected.
- 5 38. A test kit for detecting cells which contain a vertebrate *hedgehog* mRNA transcript, comprising a probe/primer of claim 36 for measuring, in a sample of cells, a level of nucleic acid encoding a vertebrate *hedgehog* protein.
- 10 39. A method of determining if a subject is at risk for a disorder characterized by unwanted cell differentiation, dedifferentiation or proliferation, comprising detecting, in a sample of tissue of said subject, the presence or absence of a genetic lesion characterized by at least one of
- 15 a mutation of a gene encoding a *hedgehog* protein, or a homolog thereof; and the mis-expression of said gene.
- 20 40. The method of claim 39, wherein detecting said genetic lesion comprises ascertaining the existence of at least one of
- 25 i. a deletion of one or more nucleotides from said gene,
ii. an addition of one or more nucleotides to said gene,
iii. an substitution of one or more nucleotides of said gene,
iv. a gross chromosomal rearrangement of said gene.
v. a gross alteration in the level of a messenger RNA transcript of said gene,
vi. the presence of a non-wild type splicing pattern of a messenger RNA transcript of said gene, and
vii. a non-wild type level of said protein.
- 30 41. The method of claim 39, wherein detecting said genetic lesion comprises
- 35 i. providing a probe/primer comprising an oligonucleotide containing a region of nucleotide sequence which hybridizes to a sense or antisense sequence of one or more of SEQ ID No:1, SEQ ID No:2, SEQ ID No:3, SEQ ID No:4, SEQ ID No:5, SEQ ID No:6, SEQ ID No:7, or naturally occurring mutants thereof or 5' or 3' flanking sequences naturally associated with said gene;
ii. exposing said probe/primer to nucleic acid of said tissue; and
iii. detecting, by hybridization of said probe/primer to said nucleic acid, the presence or absence of said genetic lesion.

183

42. The method of claim 39, wherein detecting said lesion comprises utilizing said probe/primer to determine the nucleotide sequence of said gene and, optionally, of said flanking nucleic acid sequences.
- 5 43. The method of claim 39, wherein detecting said lesion comprises utilizing said probe/primer to in a polymerase chain reaction (PCR).
44. The method of claim 39, wherein detecting said lesion comprises utilizing said probe/primer in a ligation chain reaction (LCR).
- 10 45. The method of claim 40, wherein the level of said protein is detected in an immunoassay.
46. A composition suitable for pharmaceutical administration comprising
- 15 (i) at least one polypeptide capable of functioning in one of either role of an agonist of at least one biological activity of a vertebrate *hedgehog* protein or an antagonist of at least one biological activity of said vertebrate *hedgehog* protein; and
- (ii) a pharmaceutically acceptable carrier.
- 20 47. The pharmaceutical composition of claim 46, wherein said polypeptide comprises an amino acid sequence selected from the group consisting of SEQ ID No:8, SEQ ID No:9, SEQ ID No:10, SEQ ID No:11, SEQ ID No:12, SEQ ID No:13, SEQ ID No:14, SEQ ID No. 34, SEQ ID No. 40 and SEQ ID No. 41.
- 25 48. A method for inducing a cell to differentiate to a neuronal cell phenotype, comprising contacting said cell with a protein of claim 1.
49. A method for modulating, in an animal, cell growth, cell differentiation or cell survival, comprising administering a therapeutically effective amount of a *hedgehog* polypeptide.
- 30 50. A method for treating a degenerative disorder of the nervous system characterized by neuronal cell death, comprising administering to a patient a therapeutically effective amount of a pharmaceutical preparation of a *hedgehog* polypeptide comprising and
- 35 amino acid sequence selected from the group consisting of SEQ ID No:8, SEQ ID No:9, SEQ ID No:10, SEQ ID No:11, SEQ ID No:12, SEQ ID No:13, SEQ ID No:14, SEQ ID No. 34, SEQ ID No. 40, SEQ ID No. 41 or homologs thereof.

184

51. The method of claim 50, wherein said therapeutically effective amount of said *hedgehog* polypeptide inhibits the de-differentiation of neuronal cells of said patient.
52. The method of claim 51, wherein said neural cell phenotype is a glial cell.
53. The method of claim 1 wherein said neural cell phenotype is a nerve cell.
54. The method of claim 50 wherein said degenerative disorder is a neuromuscular disorder.
55. The method of claim 50, wherein said degenerative disorder is an autonomic disorder.
56. The method of claim 50, wherein said degenerative disorder is a central nervous system disorder.
57. The method of claim 50, wherein said degenerative disorder is selected from a group consisting of Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, Pick's disease, Huntington's disease, multiple sclerosis, neuronal damage resulting from anoxia-ischemia, neuronal damage resulting from trauma, and neuronal degeneration associated with a natural aging process.
58. The method of claim 50, further comprising administering a therapeutically effective amount of a growth factor having neurotrophic activity is administered to said patient.
59. The method of claim 58, wherein said growth factor is selected from a group consisting of a nerve growth factor, ciliary neurotrophic growth factor, schwannoma-derived growth factor, glial growth factor, striatal-derived neurotrophic factor, platelet-derived growth factor.
60. A peptidomimetic of a portion of a vertebrate *hedgehog* protein, which peptidomimetic modulates at least a portion of the biological activities of said vertebrate *hedgehog* protein.
61. A differentiated cell generated in cell culture by contacting an undifferentiated cell from a vertebrate organism with a *hedgehog* polypeptide.
62. The differentiated cell of claim 61, which differentiated cell is a neuronal cell.
63. The differentiated cell of claim 62, which neuronal cell is a dopaminergic cell.

DROSOPHILA HEDGEHOG
CHICKEN HEDGEHOG-A
CHICKEN HEDGEHOG-B

R	C	K	E	K	L	N	V	L	A	Y	S	V	M	N	E	W	P	G	I	R	L	L	V	T
R	C	K	E	R	V	N	S	L	A	I	A	V	M	H	M	W	P	G	V	R	L	R	V	T
R	C	K	D	K	L	N	A	L	A	I	S	V	M	N	Q	W	P	G	V	K	L	R	V	T

DROSOPHILA HEDGEHOG
CHICKEN HEDGEHOG-A
CHICKEN HEDGEHOG-B

E	S	W	D	E	D	Y	H	H	G	Q	E	S	L	H	Y	E	G	R	A	V	T	I	A	T
E	G	W	D	E	D	G	H	H	L	P	D	S	L	H	Y	E	G	R	A	L	D	I	T	T
E	G	W	D	E	D	G	H	H	S	E	E	S	L	H	Y	E	G	R	A	V	D	I	T	T

DROSOPHILA HEDGEHOG
CHICKEN HEDGEHOG-A
CHICKEN HEDGEHOG-B

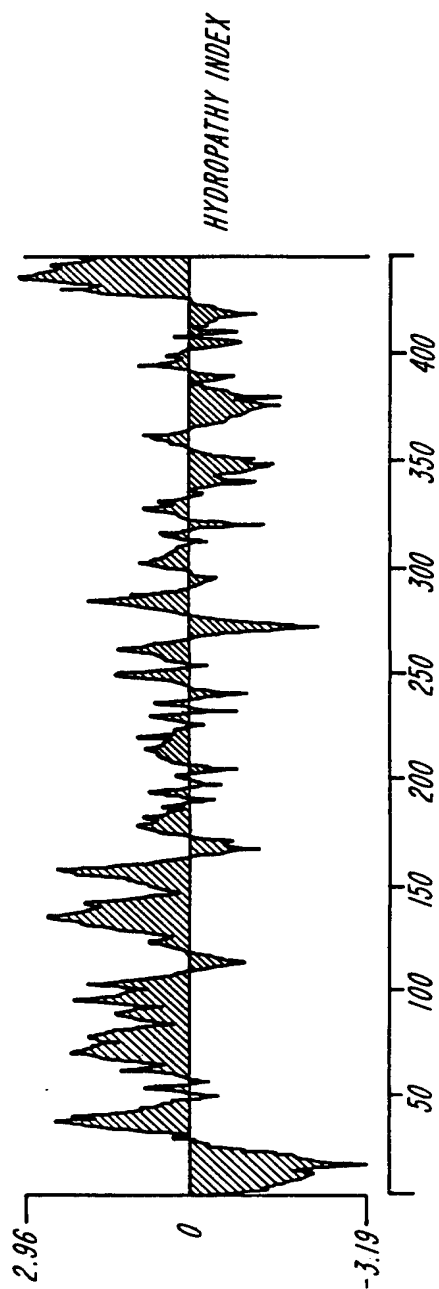
S	D	R	D	Q	S	K	Y	G	M	L	A	R	L	A	V	E	A	G	F	D	W	V
S	D	R	D	R	H	K	Y	G	M	L	A	R	L	A	V	E	A	G	F	D	W	V
S	D	R	D	R	S	K	Y	G	M	L	A	R	L	A	V	E	A	G	F	D	W	V

FIGURE 1

[illegible]

FIGURE 2

3/21

*FIG. 3*

4/21

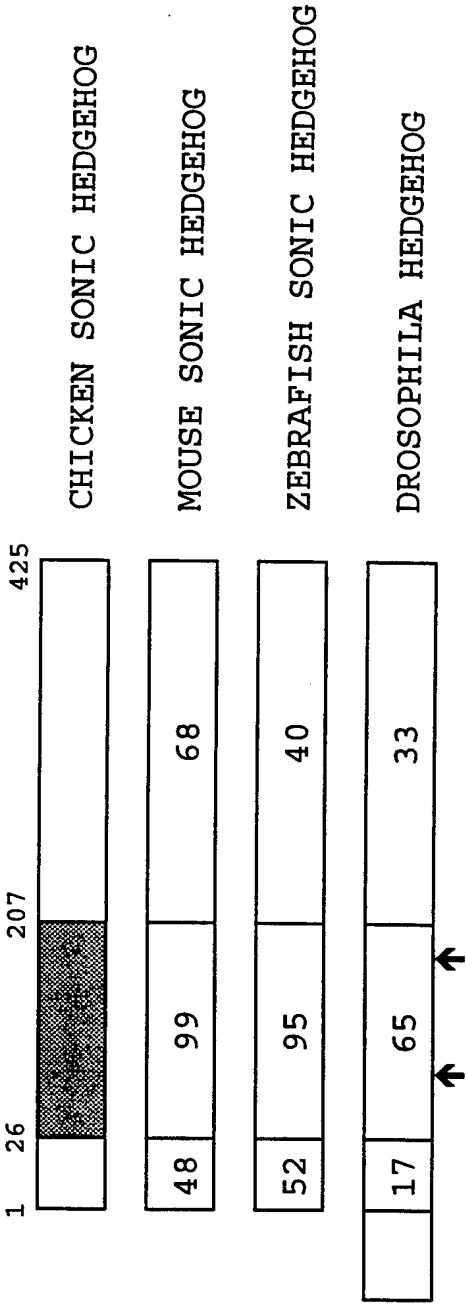


FIGURE 4

1	b-hh	MDNHSSVPWA	SAASVTCLSL	DAKCHSSSSS	SSSKSAASSI	SAIPQEETQT
	M-Dhh
	M-Ihh
	M-Shh
	C-Shh
	Z-Shh
51	D-hh	MRHIAHTQRC	LSRLTSLVAL	LLIVLPMVFS	PAHSCGPGRG	LGRHR...AR
	M-DhhMALPASLL	PLCCCLALLAL	SAQSCGPGRG	PVGRRRYVRK
	M-Ihh
	M-Shh	MLLLLARCFI	VILASSLLVC	PGLACGPGRG	FGKRRH...PK
	C-ShhMV	EMLLLTRILL	VGFIGALLVS	SGLTCGPGRG	IGKRRH...PK
	Z-ShhMRLLTRVLL	VSLLTLSLVV	SGLACGPGRG	YGRRRH...PK
101	D-hh	NLYPLVLXQT	IPNLSEYTNL	ASGPLEGVIR	RDSPKPKDLV	PNYNRDILFR
	M-Dhh	QLVPLLYKQF	VPSMPERTLG	ASGPAEGRVT	RGSERFRLDV	PNYNPDIIFK
	M-IhhERPKELT	PNTNPDIIFK
	M-Shh	KLTPLAYKQF	IPNVAEKTGL	ASGRYEGKIT	RNSERPKELT	PNTNPDIIFK
	C-Shh	KLTPLAYKQF	IPNVAEKTGL	ASGRYEGKIT	RNSERPKELT	PNTNPDIIFK
	Z-Shh	KLTPLAYKQF	IPNVAEKTGL	ASGRYEGKIT	RNSERPKELT	PNTNPDIIFK
151	D-hh	DEEGTGADRL	MSKRCCKEKL	VLAISVHNH	PGIRLLVTES	WDEDYHHGQE
	M-Dhh	DEENSGADRL	MTQRCCKEKL	ALAIASVHNH	PGVRLRVTEG	WDEDGHHAQD
	M-Ihh	DEENTGADRL	MTQRCCKDRL	SLAISVHNH	PGVKLRVTEG	RDEDGHHSEE
	M-Shh	DEENTGADRL	MTQRCCKDRL	ALAIASVHNH	PGVRLRVTEG	WDEDGHHSEE
	C-Shh	DEENTGADRL	MTQRCCKDRL	ALAIASVHNH	PGVKLRVTEG	WDEDGHHSEE
	Z-Shh	DEENTGADRL	MTQRCCKDRL	SLAISVHNH	PGVKLRVTEG	WDEDGHHFEE

FIGURE 5 (1)

201	D-hh	SLHYEGRAVT	IATSDRDQSK	YGMLARLAVE	AGPDWVSYS	RRHIYCSVKS
	M-Dhh	SLHYEGRALD	ITTSDRDRNK	YGLLARLAVE	AGPDWVYYS	RNHIHVSVKA
	M-Ihh	SLHYEGRAVD	ITTSDRDRNK	YGLLARLAVE	AGPDWVYYS	KAHVHC SVKS
	M-Shh	SLHYEGRAVD	ITTSDRDRSK	YGMLARLAVE	AGPDWVYYS	KAHHC SVKA
	C-Shh	SLHYEGRAVD	ITTSDRDRSK	YGMLARLAVE	AGPDWVYYS	KAHHC SVKA
	Z-Shh	SLHYEGRAVD	ITTSDRDKSK	YGTLSRLAVE	AGPDWVYYS	KAHHC SVKA
251	D-hh	DSSISSHVHG	CPTPESTALL	ESGVRKPLGE	LSIGDRVLSM	TANGQAVYSE
	M-Dhh	DNSLAVRAGG	CFPGNATVRL	RSGERKGLRE	LHRGDWVLA	DAAGR VVPTP
	M-Ihh	EHSAAAKTGG	CFPAGAQVRL	ENG ERVALSA	VKPGDRVLAM	GEDGTPTFSD
	M-Shh	ENSVAAKSGG	CFPGSATVHL	EQGGTKLVKD	LRPGDRVLA	DDQGRLLYSD
	C-Shh	ENSVAAKSGG	CFPGSATVHL	EHGGTKLVKD	LSPGDRVLA	DADGRLLYSD
	Z-Shh	ENSVAAKSGG	CFPGSALVSL	QDGGQKAVKD	LNPGDKVLA	DSAGNLVFS
301	D-hh	VILEFMDRNLE	QMONEVQLHT	DGGAVLTVT	PAHLVSVWQPESQ
	M-Dhh	VLLFLDRDLQ	RRASFVAVET	ERPPRKLLLT	PWHLVFAAR	...GPAPAPG
	M-Ihh	VLIPLDREP	RLRAFQVIET	QDP RRLALT	PAHLLEFIADN	HTE.....PAA
	M-Shh	FLTFLDRDEG	AKKVFYVIET	LEPRERLLLT	AAHLLEFVAP	HNDSGPTPGP
	C-Shh	FLTFLDRMDS	SRKLFPYVIET	RQPRARLLLT	AAHLLEFVAPQ	HNQSEATGST
	Z-Shh	FIMFTDRDST	TRRVFYVIET	QEPVEKITLT	AAHLLEFVLDN	STEDLHTMT
351	D-hh	KLTFVEADRI	EEKNQVLV..	RDVETGELRP	QRVVKVG.SV	RSKGVVAPLT
	M-Dhh	DFAPVFAARRL	RAGDSVLA..	..PGDALQP	ARVARVA.RE	EAVGVFAPLT
	M-Ihh	HFRATFASHV	QPGQYVLV..	..SGVPGLQP	ARVAASV.TH	VALGSYAPLT
	M-Shh	S..ALFASRV	RPGQRVYVVA	ERGDDRLLP	AAVHSVTLRE	EEAGAYAPLT
	C-Shh	SGQALFASNV	KPGQRVYVLG	E..GGQQLLP	ASVHSVSLRE	EASGAYAPLT
	Z-Shh	...AAYASSV	RAGQKVMVD	DSGQLKSVIV	QRIYT.....E	EQRGSFAPVT

FIGURE 5 (2)

6/21

7/21

401	D-hh	REGTIVNSV	AASCYAVINS	QSLAHWGLAP	MRLSTLEAW	LPAKEQLHSS
	M-Dhh	AHGTLLVNDV	LASCYAVLES	HQWAHRAFAF	LRLHALGAL	LP.....
	M-Ihh	RHGTLVVEDV	VASCFAAVAD	HHLAQLAFWP	LRLFPSL...
	M-Shh	AHGTILINRV	LASCYAVIEE	HSWAHRAFAF	FRLAHALLAA	LAPARTDGGG
	C-Shh	AQGTILINRV	LASCYAVIEE	HSWAHWAFAF	FRLAQGLLAA	ICP.....
	Z-Shh	AHGTIVVDRI	LASCYAVIED	QGLAHLAFAP	ARLYYVVSFF	LSP.....
451	D-hh	PKVV.....	...SSAQQQN	GIHWYANALY	KVKDYVLPQS	WRHD*
	M-DhhGGAVQPT	GMHWYSRLLY	RLAEELMG*	
	M-Ihh	•AWGSWTPSE	GVHSYPQMLY	RLGRLLLEES	TFHPLGMSG
	M-Shh	CGSIPAAQSA	TEARGAEPTA	GIHWYSQLLY	HIGTWLLDSE	RMHPLGMAVK
	C-Shh	DGAIPTA...ATTIT	GIHWYSRLLY	RIGSWVLDGD	ALHPLGMVAP
	Z-Shh	KTPAVGPMRL	YNRRGSTGTP	GSC.....H	QMGTWLLDSN	MLHPLGMSVN
501						
	M-Ihh	GS*				
	M-Shh	SS*				
	C-Shh	AS*				
	Z-Shh	SS*				

FIGURE 5 (3)

[illegible][illegible]

H A Q D S L H Y E G G R A L D I T T S D R D R N K Y G L L A R L A V E A G F D W V Y Y E S R N H I H V S V K A D
 H H S E E S L H Y E G G R A V D I T T S D R D R N K Y G L L A R L A V E A G F D W V Y Y E S K A H V H C S V K S E
 H H S E E S L H Y E G G R A V D I T T S D R D R N K Y G L L A R L A V E A G F D W V Y Y E S K A H V H C S V K S E
 H H S E E S L H Y E G G R A V D I T T S D R D R S K Y G M L A R L A V E A G F D W V Y Y E S K A H I H C S V K A E
 H H S E E S L H Y E G G R A V D I T T S D R D R S K Y G M L A R L A V E A G F D W V Y Y E S K A H I H C S V K A E
 H H S E E S L H Y E G G R A V D I T T S D R D R S K Y G M L A R L A V E A G F D W V Y Y E S K A H I H C S V K A E
 H H F E E S L H Y E G G R A V D I T T S D R D K S K Y G T L S R L A V E A G F D W V Y Y E S K A H I H C S V K A E
 H H X X S L H Y E G G R A X D I T T S D R D X X K Y G X L X R R L A V E A G F D W V Y Y E S X X H X H X S V K X X

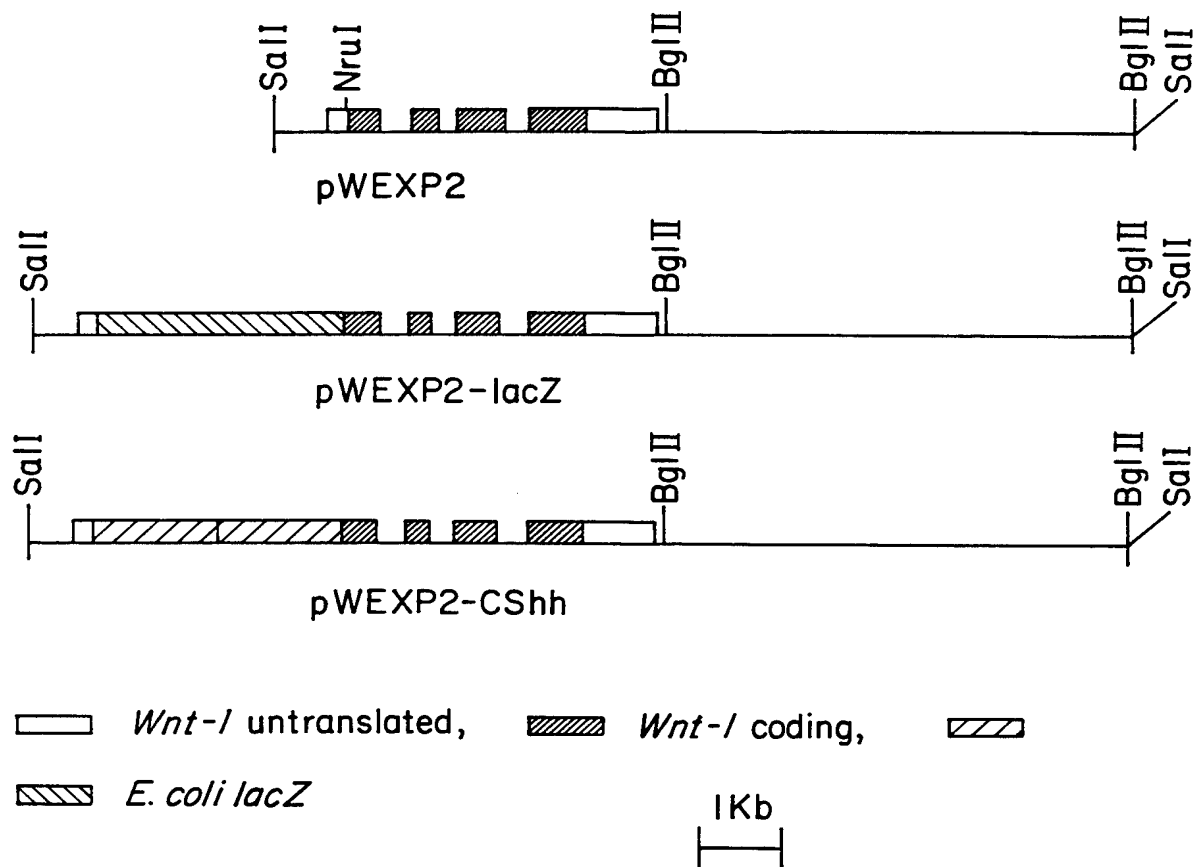
Figure 5B

9/21

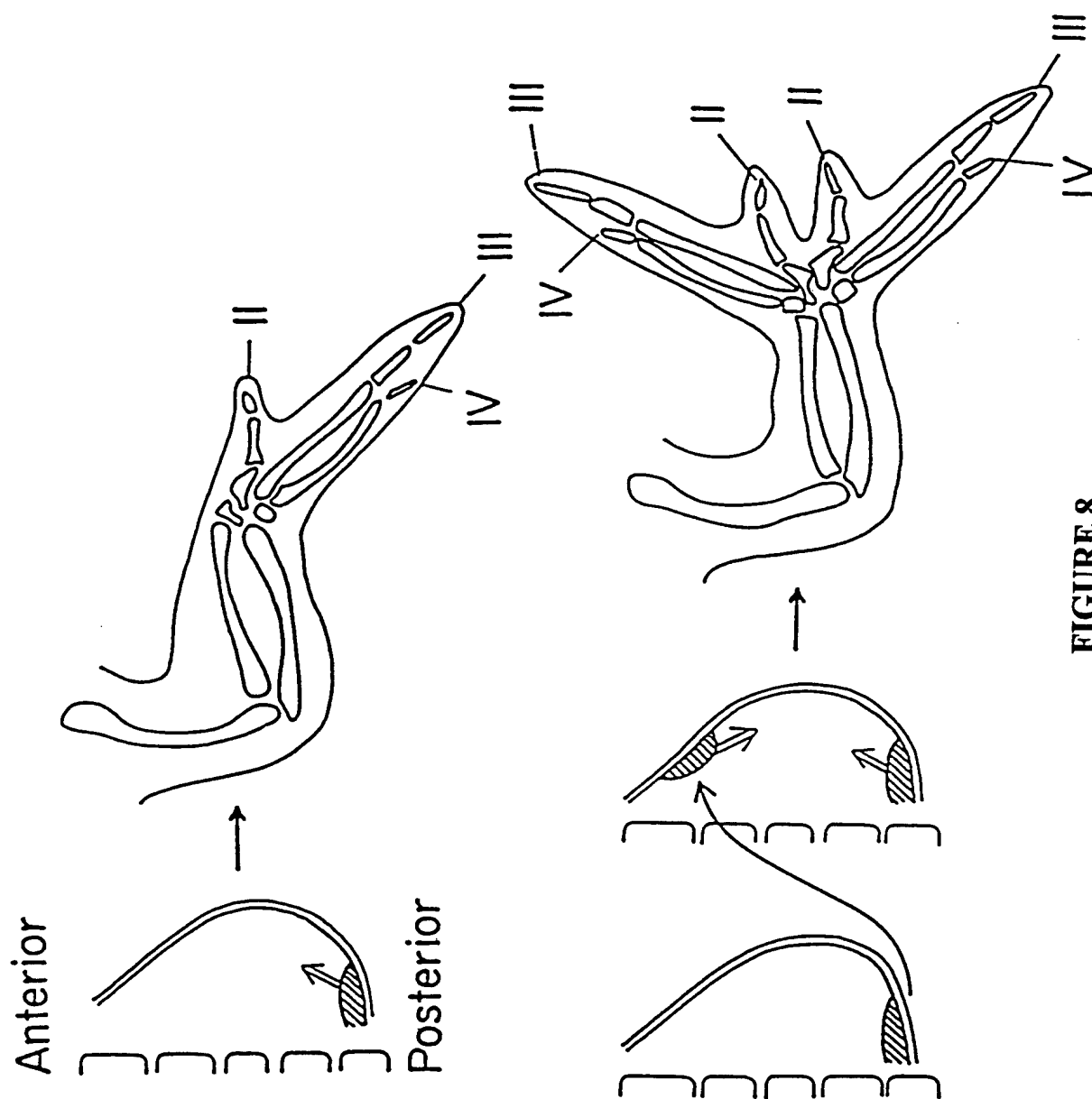
	M-Dhh	M-Ihh	C-Shh	Zf-Shh	D-hh
M-Shh	61 (77)	63 (78)	84 (92)	68 (80)	48 (64)
M-Dhh		58 (75)	61 (77)	54 (71)	51 (68)
M-Ihh			64 (78)	61 (75)	48 (68)
C-Shh				68 (80)	49 (64)
Zf-Shh					47 (64)

FIGURE 6

10/21

**FIG. 7**

11/21



12/21

```

hh 1 MDNHSSVPWASAAVTCLSLDAKCHSSSSSSSSKSAASSISAIPQEETQT
shh .....

hh 51 MRHIAHTORCLSRYLTSLVALLLVLPMVFSPAHSYCGPGRGLGRHR.ARNYL
shh 1 .....MRLLTRVLLVSLYLTSLVVS.GLACGPGRGYRRRHPPKKL

hh 100 YPLVLKQITIPNLSEYTNYNSASGPLEGVIRRDSPKFKDLVPNYNRDILFRDE
shh 40 TPLAYKQFIPNVAEKTYLGASGRYEGKITRNSERFKELTPNYNPDIIFKDE

hh 150 EGTGADRLMSKRCYCKEKLNVLAYSVYMNEWPGIRLVVYTESWDEDYHHGQESL
shh 90 ENTGADRLMTQRCYCKDKLNSLAISVMNHWPYGVKLRVTEGWDYEDGHHFEESL

hh 200 HYEGRYAVTIATSDRDQSKYYGMLARLAVEAGFDWVSYYVSRYRHIYCSVKSDS
shh 140 HYEGRYAVDITTSYDRDKSKYYGTLSRLAVEAGFDWVSYYESKAHIHCSVKAEN

hh 250 SISSHVHGCFYTPETALLESYGVKPLGELSIGDRVLYSMTANGQAVYSEVI
shh 190 SVAAKSGGCFYPGSALVSLQDGGQKAVKDLNPGDKVLAADSAGNLVFSDFI

```

FIGURE 9A (1)

15/21

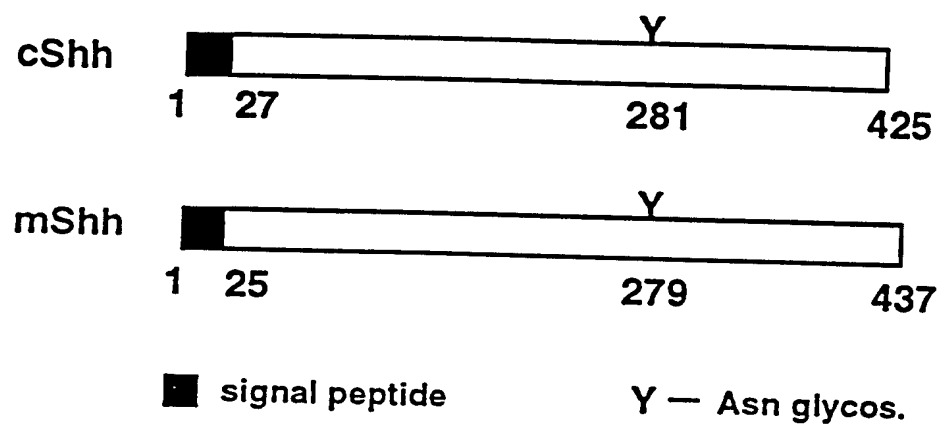


FIGURE 11

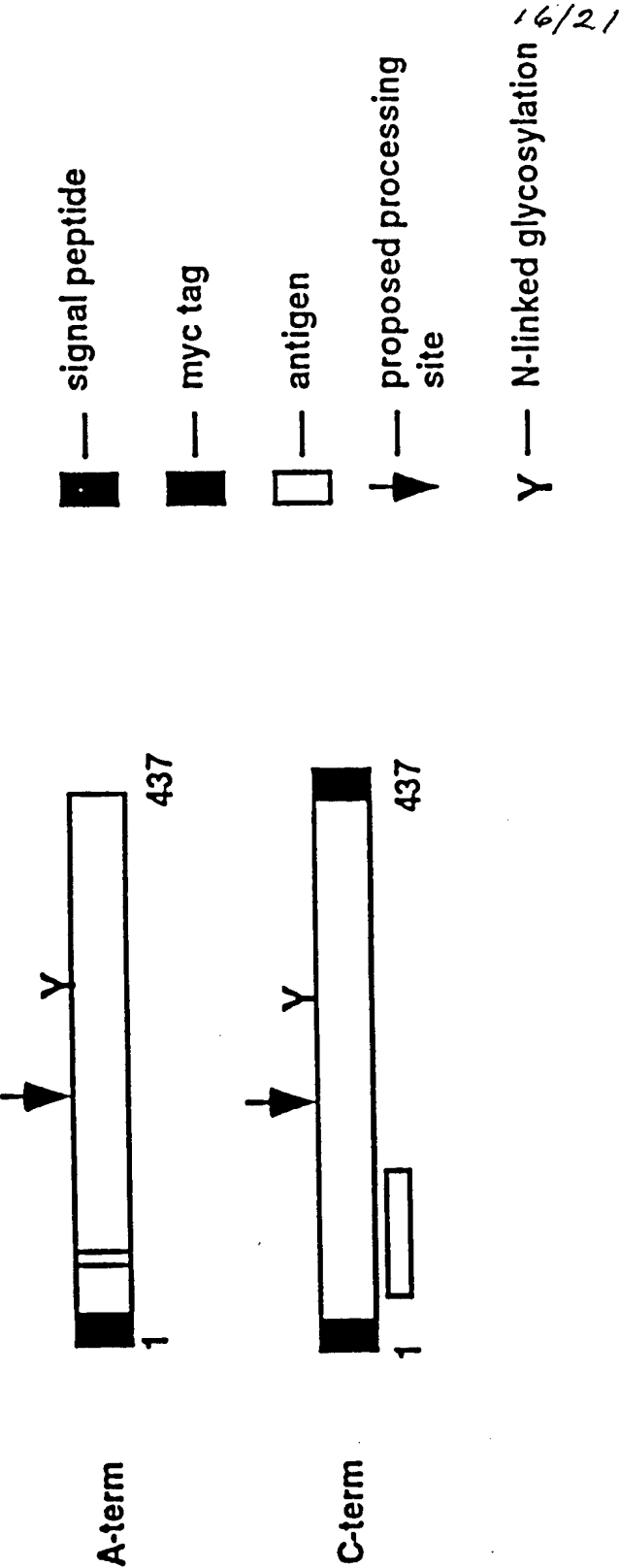


FIGURE 12

17/21

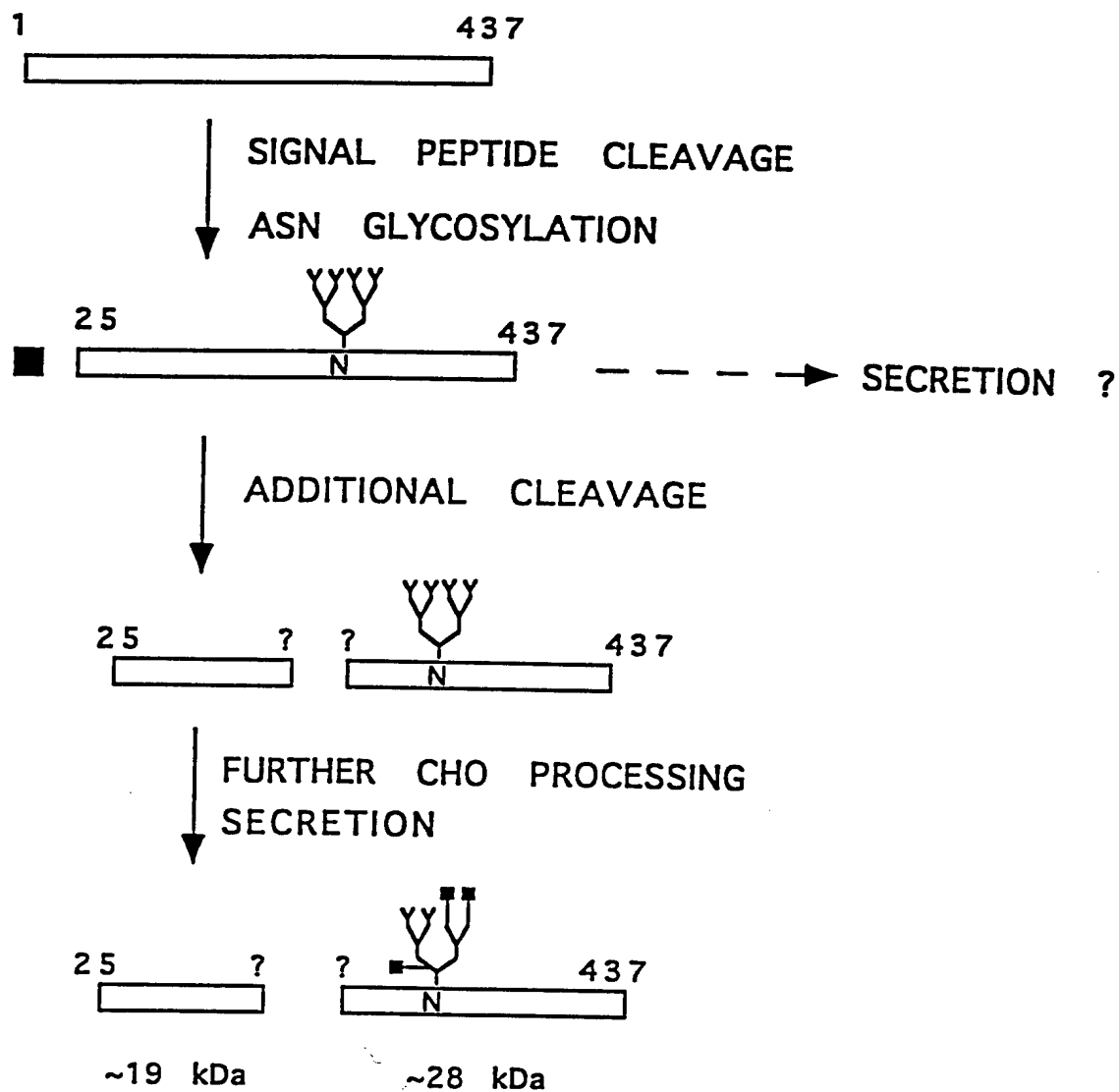


FIGURE 13

18/21

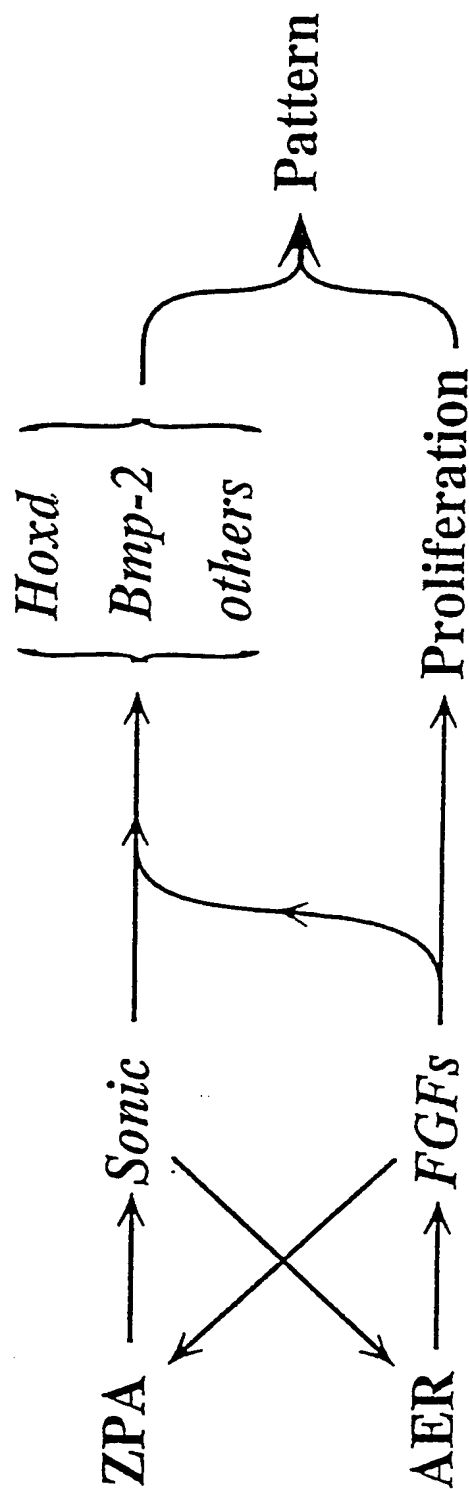


FIGURE 14

19/21

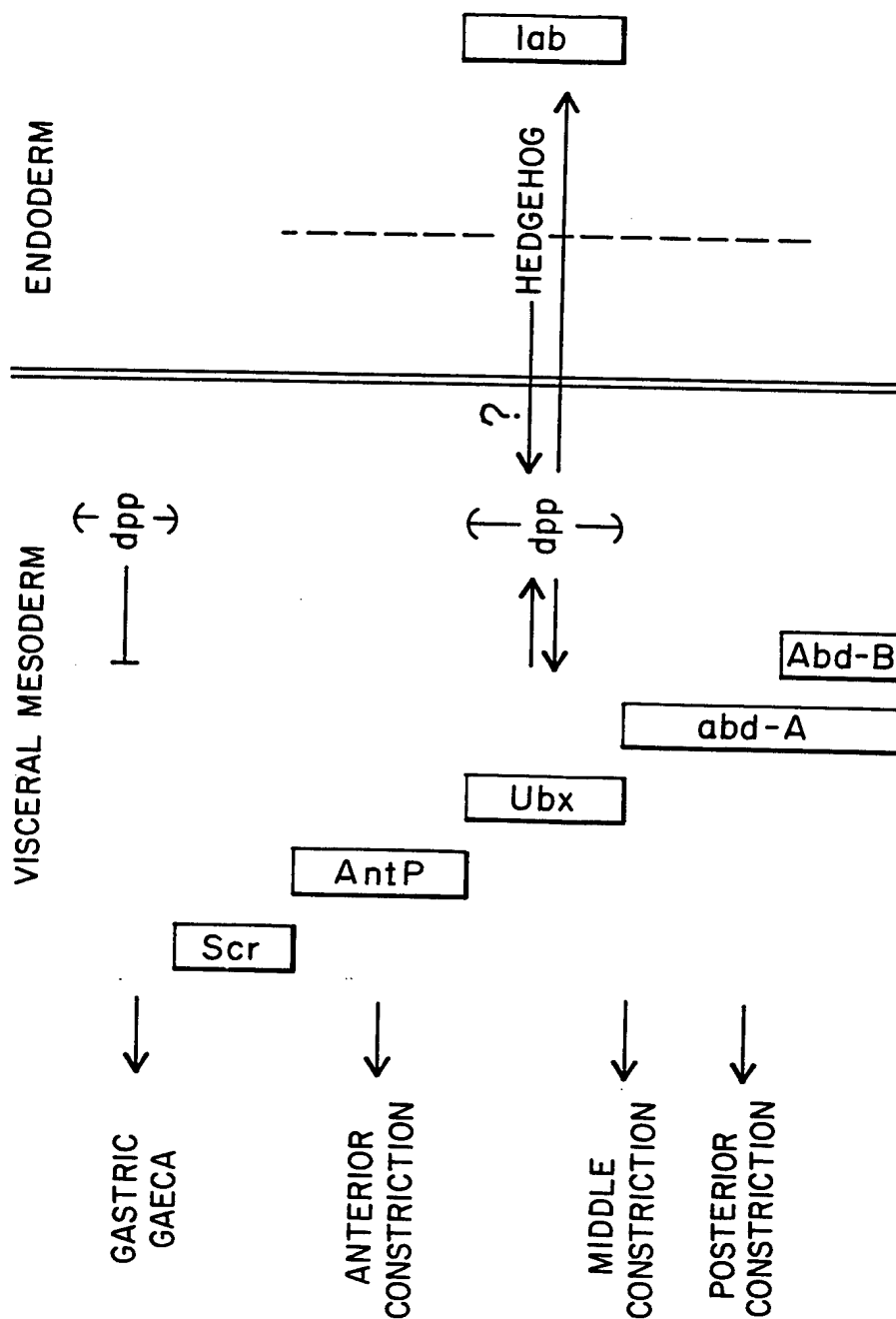


FIG. 15A

20/21

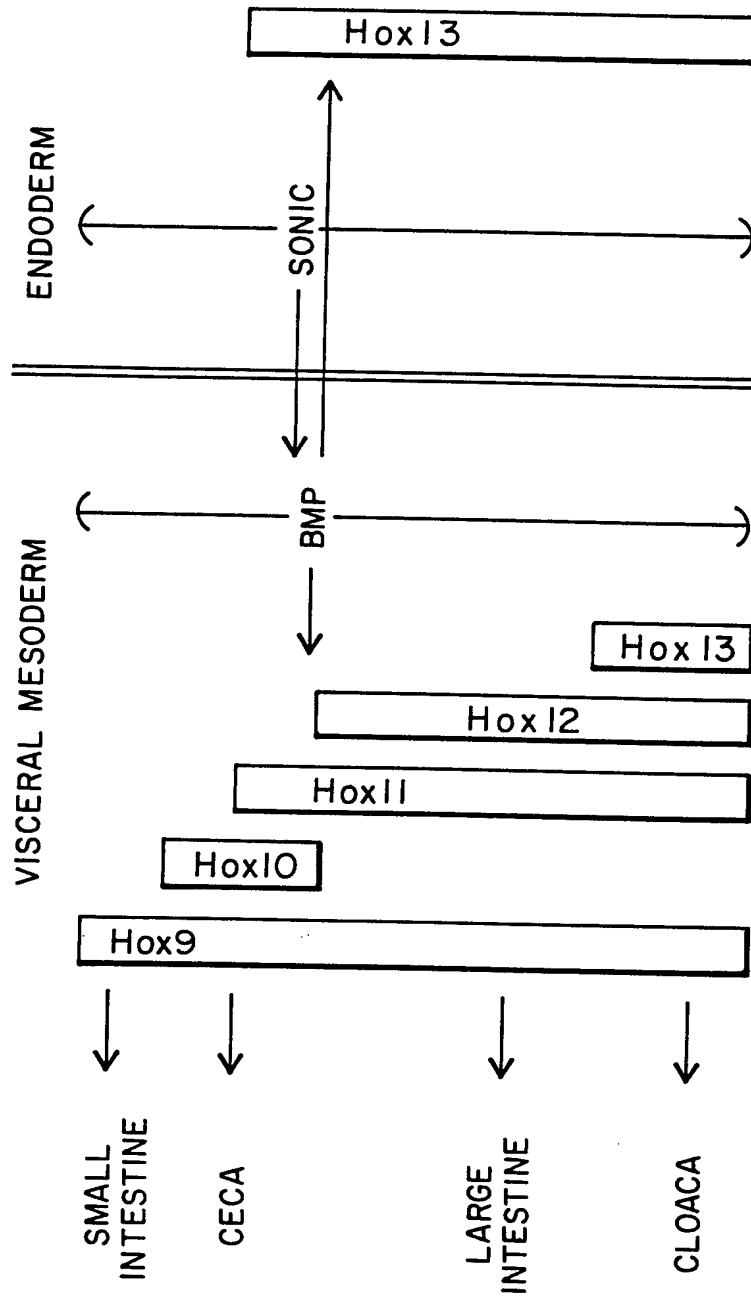


FIG. 15B

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 94/14992

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/12 C07K14/46 C07K14/435 A61K48/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	DEVELOPMENT, vol.0, no.SUPP, 1994 pages 43 - 51 FIETZ, M. ET AL. 'The hedgehog gene family in Drosophila and vertebrate development' see the whole document ---	1,2,7,8, 16,17, 24-26, 36,37
P,X	CELL., vol.75, no.7, 31 December 1993, CAMBRIDGE, NA US pages 1401 - 1416 RIDDLE, R.D. ET AL. 'Sonic hedgehog mediates the polarizing activity of the ZPA' see the whole document --- -/--	1,2,7,8, 16

☒ Further documents are listed in the continuation of box C.☐ Patent family members are listed in annex.

* Special categories of cited documents :

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- "&" document member of the same patent family

Date of the actual completion of the international search

8 June 1995

Date of mailing of the international search report

20.06.95

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
Fax (+31-70) 340-3016

Authorized officer

Chambonnet, F

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 94/14992

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	CELL., vol.75, no.7, 31 December 1993, CAMBRIDGE, MA US pages 1417 - 1430 ECHELARD, Y. ET AL. 'Sonic hedgehog a member of a family of putative signaling molecules, is implicated in the regulation of CNS polarity' see the whole document ---	1-3,7-9, 16,25-27
P,X	CELL., vol.76, no.4, 25 February 1994, CAMBRIDGE, MA US pages 761 - 775 ROELINK, H. ET AL. 'Floor plate and motor neuron induction by vhh-1, a vertebrate homolog of hedgehog expressed by the notochord' see the whole document -----	1,7-9, 14-16, 22,25, 29-33, 36,49

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 94/ 14992

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 50-59 and partially 48-49, as far as it concerns a method in vivo of treatment, are directed to a method of treatment of the human/animal body the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.